PHD

Analytical and protein-binding studies of cephalosporin antibiotics

Alhadiyah, Badreddin M. Hashim

Award date:
1989

Awarding institution:
University of Bath

ANALYTICAL AND PROTEIN-BINDING STUDIES

OF

CEPHALOSPORIN ANTIBIOTICS.

Thesis Submitted by :

BADREDDIN M. HASHIM ALHADIYAH B.Pharm, M.Sc.,

for the degree of Doctor of Philosophy

of the University of Bath.

1989

This reasearch has been carried out in the School of Pharmacy and Pharmacology of University of Bath under the joint supervision of Dr. A. F. Casy and Dr. S. K. Branch.

COPYRIGHT

SIGNED:

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

IN THE NAME OF ALLAH
THE MERCIFUL THE COMPASSIONATE

اَلْحَمْدُ لِلّٰهِ رَبِّ الْعٰلَمِيْنَ

وَالصَّلٰوةُ وَالسَّلَامُ عَلٰى سَيِّدِنَا مُحَمَّدٍ خَاتَمِ النَّبِيِّيْنَ

Dedication

To the beloved memory of my mother

To my wife, Suoad, for her unfailing support, patience and understanding.

To our children, Eiman, Khalid, Ehsan and Omer.

Humbly dedicated also to every member of my family for their continuous interest and moral support.

ACKNOWLEDGEMENT

I would like to express my grateful thanks to King Faisal Foundation, Saudi Arabia, for financial support.

My sincere appreciation and gratitude to my supervisors Dr. Alan F. Casy and Dr. Sarah K. Branch for their great interest, guidance, constant encouragement, and extremely helpful discussions and advice throughout the course of this work.

Appreciation is also extended to Mr. Harry Hartell and Mr. Dave Wood for their help in operating the Jeol FT NMR GX270 spectrometer and for securing part of the H and C NMR spectra, to Mr. Don Perry, Mr. Kevin Smith and Mr. Richard Sadler for their skilled technical assistance.

This thesis was typed on GOULD Computer Word Processor with UNIX Software in Bath University Computer Services (BUCS), with the appreciated help of members of staff in the Computer Operations especially Mr. John Gardiner, and my colleagues, Nawi, Nasreddine, Benboubetra and Tahseen.

Last, but not least, thanks to all my friends and members of staff of Department of Pharmaceutical Chemistry who made my time in Bath most memorable.

SUMMARY

A brief general introduction to cephalosporin antibiotics is presented in Chapter One, with particular reference to the structural elucidation of cephalosporin C which is considered as the parent substance from which the first cephalosporins of clinical importance were derived. Attention is also drawn to the importance of stability and degradation studies of the pure antibiotic compound to pharmaceutical industry.

In Chapter Two, the synthesis and characterisation of some reference standards and related compounds is reported, together with lists of suppliers and chemical structures of the cephalosporin antibiotic samples employed in the present work.

Extensive use of high-resolution ^{1}H (270 and 400 MHz) and ^{13}C (67.8 MHz) NMR has been made to study the general and specific spectral features of some cephalosporins and their derivatives. The application of NMR spectra to the identification of these antibiotics is given in Chapter Three. The data secured are also employed for confirmation of structure of esters obtained from industry and those compounds prepared in our own laboratories. Some quaternary carbon atoms have been differentiated by the use of spin-lattice relaxation time (T_1) measurements.

One of the aims of this thesis is to assess the potential of ^{1}H NMR spectroscopy in providing information about the degradation of cephalosporins under various experimental conditions. Chapter Four describes the results of this study; efforts were made to determine the structure of the degradation products. A ^{1}H NMR study of the effect of β-lactamase enzyme on a group of penicillins was also carried out, and the results are presented and discussed as a supplement to Chapter Four.

In Chapter Five, results obtained for the binding of cephalosporin antibiotics and derivatives to serum albumin using spectrofluorimetric and spin-lattice relaxation measurement techniques are reported and discussed.

CONTENTS

CHAPTER ONE

Introduction

The cephalosporin antibiotics first appeared in clinical practice in the late 1940s. They represent a large group of closely related semi-synthetic compounds derived from cephalosporin C, a natural antibiotic produced by the mould Cephalosporium acremonium. They are bactericidal, with a wide spectrum of activity and, similarly to the penicillins, they act by inhibiting synthesis of the bacterial cell wall. Because of their relatively low toxicity to man, exhibiting no reaction in some patients allergic to penicillins and relative lack of dangerous side effects, they quickly assumed a major role in chemotherapy. At present, several derivatives of cephalosporins have been reported in the literature. The latest edition of the Martindale Extra Pharmacopeia [1] (1989) lists 41 cephalosporins in clinical use.

1.1 History of the cephalosporins

In 1945, after the discovery of the chemotherapeutic properties of penicillin, an antibiotic-producing species of cephalosporium was isolated from a sewage outfall in Sardinia[2]. Then, in 1948, further studies on this organism in Oxford showed it to produce several antibiotics, one of which was penicillin N.

Five years later, this penicillin was shown to be contaminated with a second substance, cephalosporin C (Fig.1.1), containing a β-lactam ring, but more resistant to hydrolysis by the enzyme β-lactamase[3].This substance was the original member of the large group of substances now classified as cephalosporins.

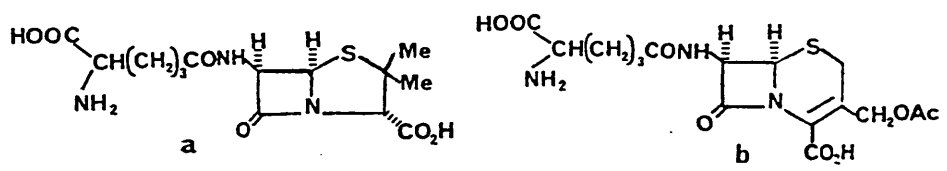


Fig. 1.1 : Structure of a) Penicillin N and b) Cephalosporin C.

By the end of 1957, larger quantities of cephalosporin C had been produced by the Medical Research Council and Glaxo laboratories in the U.K. After a detailed chemical study, Abraham and Newton published the structure of cephalosporin C in 1961[4], which was confirmed with an X-ray crystallographic study[5].

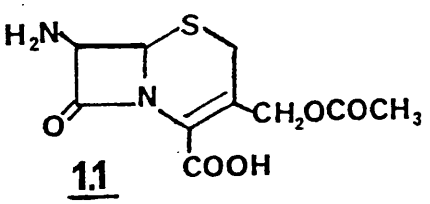
The isolation of the nucleus of cephalosporin C, 7-aminodesacetoxycephalosporanic acid (7-ADCA), in 1960s[6,7], enabled pharmaceutical manufacturers (Lilly, Glaxo, Squibb, Fujisawa etc..) to produce many thousands of cephalosporins by various derivatization procedures.

Later, in the 1970s, widely-distributed cephalosporin-producing micro-organisms were found[8]. More recently, cephalosporins have been obtained from bacteria of the Flavobacterium species[9].

These developments opened the way to the preparation of large numbers of semisynthetic cephalosporins for evaluation as therapeutic agents. The first clinically important analogue to be described was cephalothin[10], which has been widely used, especially in the USA. The first cephalosporin to be used in the United Kingdom was cephaloridine, which has the same group at position 7 as cephalothin but with methylpyridinium replacing acetoxymethyl(CH_2 OCOMe) at position 3.

1.2 Definition, stereochemistry and nomenclature of the cephalosporin antibiotics

Cephalosporins are a series of compounds containing the 7-amino-cephalosporanic acid [7-amino-3- (hydroxymethyl)-8-oxo-1-thia-5-azabicyclo[4.2.0]- oct-3-ene-4-carboxylic acid acetate] nucleus 1.1 :



1.1

Fig. 1.2 illustrates the essential structural features of the naturally occuring forms of the cephalosporin antibiotics.

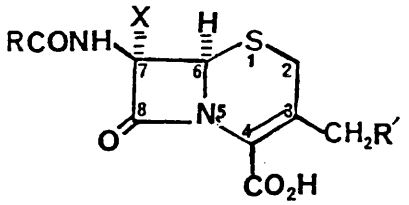


Fig. 1.2 : Cephalosporin antibiotics : a) Cephalosporins, X=H.
b) Cephamycins, X=OCH_3.

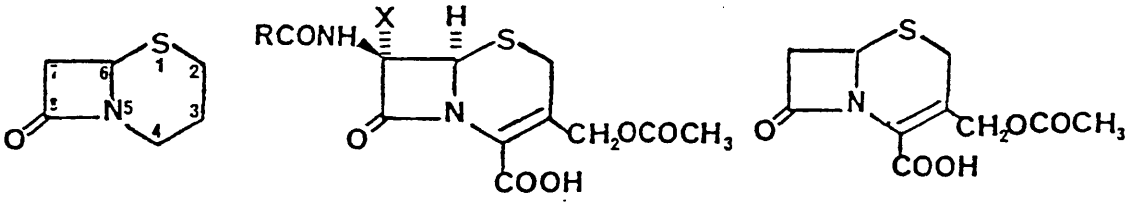
The molecular framework is a 4-membered β-lactam ring, which is fused through the nitrogen and the adjacent tetrahedral carbon atom (C-6) to a second heterocyclic ring, a six-membered dihydrothiazine ring. The compounds have a carboxyl group on the carbon atom attached to the β-lactam nitrogen (C-4), and an acylated amino group on the carbon atom adjacent to the carbonyl carbon of the β-lactam ring (C-7).

The cephalosporins and the cephamycins have the same basic structure except that the later has a methoxy group instead of hydrogen on the amide-bearing carbon-7.

The stereochemistry of the β-lactam ring of the cephalosporins involves asymmetric centres at C-6 and C-7[5,11,12], and the absolute configurations of these centres are known to be 6-R and 7-R. The fused rings are not coplanar but are folded along the C-6 to N-5 bond to give a butterfly-like molecular shape. The configuration of the fused ring is such that the hydrogen on the carbon atom at the ring junction(C-6) is cis to the hydrogen on the amide-bearing carbon atom(C-7)[13]. For the description of the cephalosporin stereochemistry, the R configuration will refer to H-6 and H-7 being behind the plane of the paper; S configuration, in front. Similarly, when substitution occurs at C-3, C-4 and C-7, α substitution denotes the group behind the plane of the paper, and β, above the plane. The stereochemical placement of the ring substituents is designated by the α and β notations. Accordingly, the hydrogens

on the β-lactam ring and the 7-methoxy group of the cephamycins are α-, and the 7-acylamino group is β.

The nomenclature of the cephalosporins and cephamycins presents some difficult problems. The Chemical Abstract formal indexing names for these antibiotics are too cumbersome for general use. For example, the official name for cephalosporin C (Fig. 1.1b, p.1) is : 3(acetoxymethyl)-7-(D-5-amino-5- carboxy-valeramido)-8-oxo-5-thia-1-azabicyclo [4.2.0]octa- 2-carboxylic acid. One simplification designates the unsubstituted bicyclic ring system of the cephalosporins, the fused dihydrothiazine-azetidinone (β-lactam) 1.2 (see Fig.1.3 below), as cepham[6]. Thus,the cephalosporins are generally 3-acetoxymethyl -7-acylamino-3-cephem-4-carboxylic acids 1.3. This nomenclature is widely used, especially in chemical literature dealing with the cephalosporins. A further simplification with narrower structural latitude is the use of the term "cephalosporanic acid" to designate the cephalosporin ring system with the substituent indicated in structure 1.4.



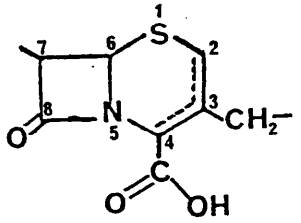
1.2 1.3 1.4

Fig.1.3

Although convenient, this naming becomes restrictive for the cephalosporins (and cephamycins) in which the 3-acetoxymethyl groupings has been replaced by other substituents.

An alternative nomenclature is the use of the Internationally approved names of cephalosporins and cephamycins rather than the more informative but cumbersome systematic chemical names, or the proprietary names, of which there are, in most instances, several for each compound.

Degradation products derived from cephalosporins are termed by Internationally approved names, e.g., desacetylcephalothin, desacetoxycephalothin, desacetylcephalothin lactone. To avoid any confusion, in this thesis, the atoms are numbered as shown below :



When the double bond is in the C3--C4 or C2--C3 position, it will be denoted as a 3-cephem (Δ^3) or 2-cephem (Δ^2) cephalosporin, respectively.

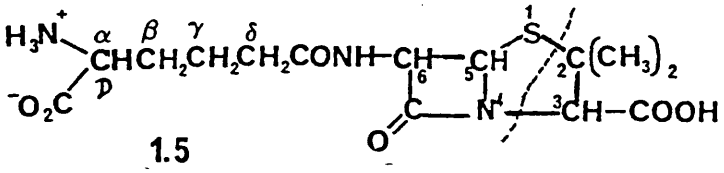
1.3 Chemistry of cephalosporins

1.3.1 Structural elucidation of cephalosporin C

(note : prior to modern physical methods)

As cephalosporin C is considered as the parent substance from which the first cephalosporins to find clinical use were derived, and as it contains the β-lactam-dihydrothiazine structure, which is the main nucleus for all cephalosporins, this section is concerned with the work which led to the determination of its chemical structure.

It was the extensive degradative, physical and physico-chemical studies of Abraham and Newton[4] that provided the chemical evidence for a definitive structure for cephalosporin C. Some of the degradative results demonstrated its resemblence to penicillin N 1.5[14,15].

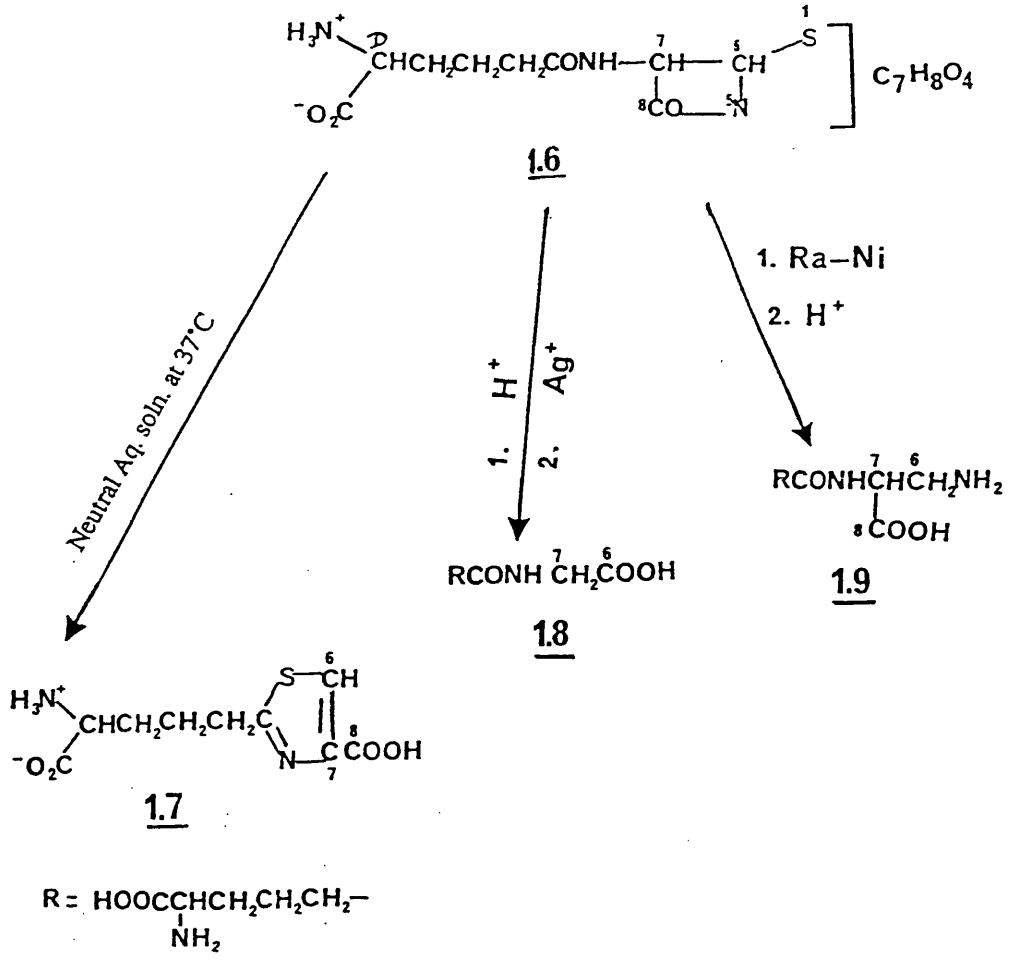


1.5

Both behaved as monoaminodicarboxylic acids. They contained D-α-aminoadipic acid linked by the δ-carbonyl to the rest of the molecule, and showed an infrared absorption band at 5.62μ, characteristic of the C=O stretching vibration of the β-lactam ring. Both yielded a mole of a CO_2 in hot dilute inorganic acid hydrolysis. After Raney nickel desulphurization, hydrolysis yielded L-alanine, valine, and glycine.

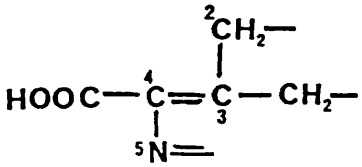
On the other hand, other degradative results showed marked differences between the two. Cephalosporin C hydrolysis did not produce penicillamine but yielded sulphur-containing fragments which contained no nitrogen. Two moles of ammonia were obtained from a hydrolysis of cephalosporin C that liberated only one mole of ammonia from penicillin N. The valine obtained from desulphurization and hydrolysis of cephalosporin C was racemic; penicillin N yielded D-valine. Cephalosporin C had an UV absorption band at 260 nm (ε=9600) absent in the penicillin. These observations, showed that the hypothesis that a modified β-lactam-thiazolidine ring system was present in cephalosporin C, would have to be abandoned.

Consideration of a number of degradation products of cephalosporin C indicated that it contained the partial structure 1.6, (Scheme 1.1, p.7) Jeffery et al.[16] (1960) reported that 2-(D-4-amino-4- carboxybutyl)thiazole-4-carboxylic acid 1.7 was formed when cephalosporin C was kept in neutral aqueous solution at 37°C. This could be accounted for by opening of the β-lactam ring, fission of the bond between the sulphur atom and the remainder of the molecule, and a nucleophilic attack of the sulphur on the amide carbon of the side chain. Acid hydrolysis of cephalosporin C produced a neutral β-acylamidoacetaldehyde fraction, which, upon oxidation with silver oxide, yielded the acylamido acetic acid 1.8. Raney-nickel desulphurization, followed by hydrolysis, degraded cephalosporin C to an acylated diaminopropionic acid 1.9 (see Scheme 1.1, p.7).



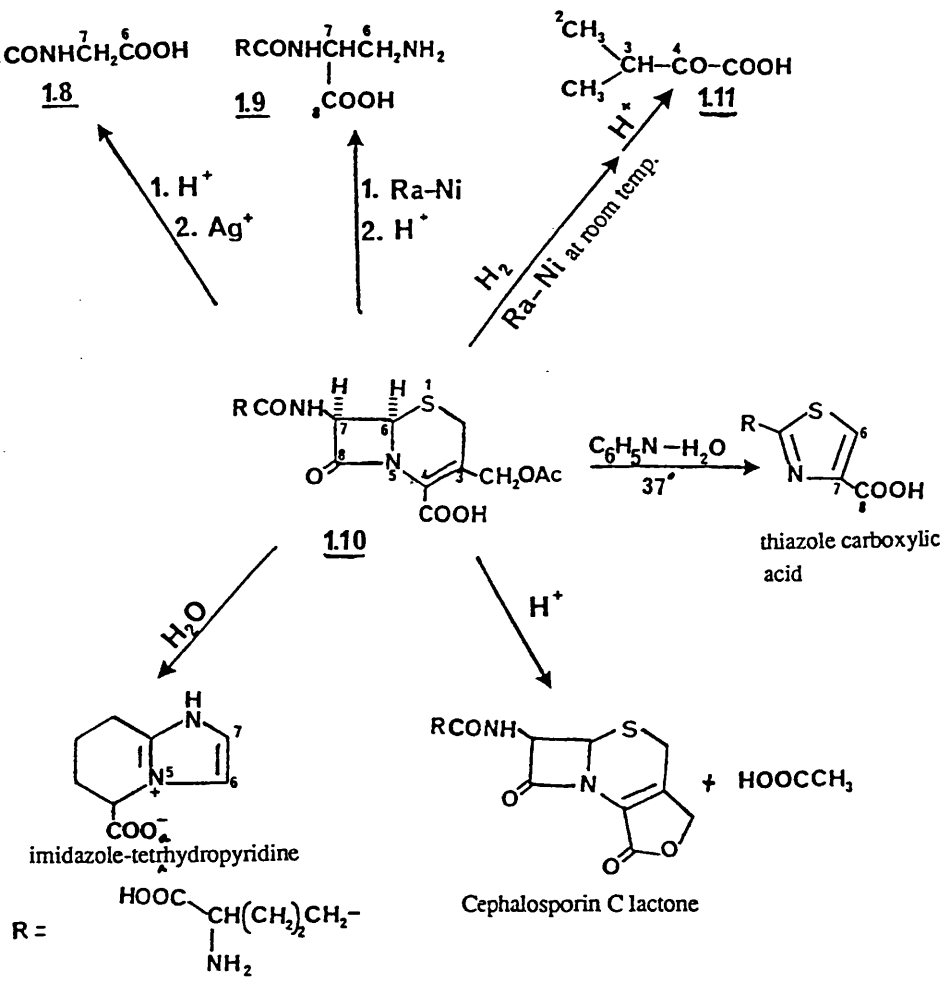
Scheme 1.1

The rest of the structure-determination of cephalosporin C proved more difficult because the dihydrothiazine ring was novel to antibiotics and was little known chemically. Nevertheless, several experiments supplied the necessary facts to prove that structure 1.10 (Scheme 2.2, p.8) satisfied all the known properties of cephalosporin C. First, hydrogenolytic hydrogenation of 1.10, followed by hydrolysis with hot acid, yielded α-oxovaleric acid 1.11. As the ^{1}H NMR spectrum of cephalosporin C failed to reveal gem-dimethyl groups (as in penicillin), and the UV absorption at 260 nm required that some sort of chromophore be associated with the nucleus outside the β-lactam ring, the investigators therefore postulated that the fragment yielding oxovaleric acid also contained the chromophore in the following form:



This finding provided five of the seven unplaced carbon atoms of the partial structure 1.6. The liberation of one mole of acetic acid from cephalosporin C by mild acid or alkaline hydrolysis established the nature of the remaining C_2 fragment. The presence of a band at 5.77 μ in the infrared spectrum of the antibiotic suggested that the acetic acid was derived from an acetoxyl group. Before final chemical proof for 1.10 was obtained, Hodgkin and Maslen[5] established by X-ray analysis that the sulphur-containing ring of cephalosporin C was six-membered and substituted by an acetoxymethyl group.

The chemical degradations which confirmed structure 1.10 were, in general,combinations of hydrogenation, desulphuration, and hydrolysis and are summarised as follows:



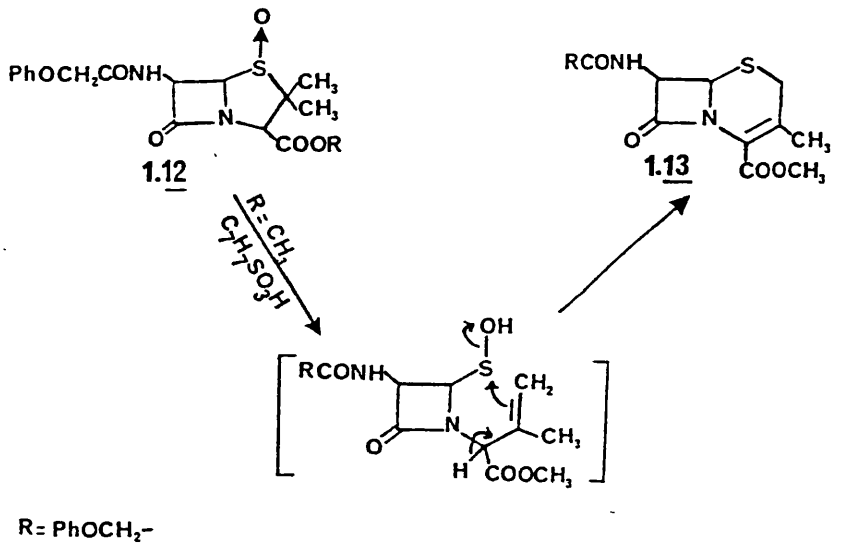
Scheme 1.2

The expected configuration, with cis hydrogen atoms attached to C_6 and C_7, was confirmed by the X-ray crystallographic analysis[5].

The isolation of the 2,4-dinitrophenylosazone of hydroxyacetone after ozonolysis of cephalosporin C and treatment of the product with Raney nickel and a solution of 2,4-dinitrophenylhydrazine in 2N HCl, was consistent with the position assigned to the double bond in structure 1.10.

1.3.2 Total chemical synthesis of cephalosporins

Most of the attempts for total synthesis of cephalosporins proved to be difficult[17–22]. This is mainly due to the unusually high degree substitution of carbon atom 3, in addition to the reactivity of the β-lactam structure, which renders these antibiotics specially vulnerable to chemical attack. Morin and co-workers[12] described a chemical procedure for the conversion of a penicillin to a desacetoxycephalosporin. They subjected phenoxymethylpenicillin sulphoxide 1.12 to Pummerer reaction conditions, p-toluene sulphonic acid in boiling xylene. The reaction mechanism involved esterification, rearrangement, and cleavage to an intermediate unsaturated sulphenic acid, in which the sulphur then attacked the terminus of the double bond to produce the six-membered ring of 1.13 :



R= $PhOCH_2$-

A detailed total chemical synthesis of cephalosporins has been given by Woodward et al.[23]. Instead of the dihydrothiazine ring, these workers chose the β-lactam ring as the initial monocycle from which to construct the cephalosporin nucleus.

1.3.3 Semisynthesis of clinically useful cephalosporins

The isolation of the cephalosporin C nucleus, 7-aminocephalosporanic acid (7-ACA, Fig. 1.4) 1.14[6], opened the way to the preparation of large numbers of semisynthetic cephalosporins for evaluation as therapeutic agents. It was used as the starting material of many new compounds [see Table 1.1, p.12] by acylation of the amine at the C_7 position using : a) acid chloride, b) mixed anhydride, or c) dicyclohexylcarbodiimide methods in aqueous or nonaqueous solvents and sodium bicarbonate or an organic base as an acid acceptor. Further chemical modifications of position 3 and 7 in cephalosporin C 1.10 (Fig. 1.4), has resulted in a series of antibiotics with different characteristics. Substitution at the 7-amino group tends to affect antibacterial action whereas at position 3 it may have more of an effect on pharmacokinetic properties.

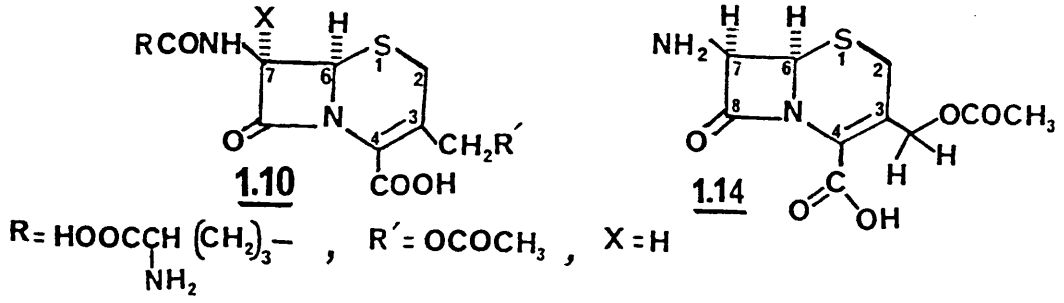


Fig. 1.4 : The cephalosporin C molecule 1.10 showing the two groups (RCO and R') in which modifications might profitably be made, and its nucleus, 7-ACA 1.14, into which new groups can readily be incorporated.

The main objectives in the synthesis of the first-generation cephalosporins at that time were :

1. Preparation of cephalosporins with high intrinsic activity not only against penicillinase-

producing bacteria, but also against a range of Gram-negative ones that were penicillin-resistant and β-lactamase-resistant.

2. Preparation of cephalosporins clinically useful when given by mouth.

The resistance of 7-α-methoxycephalosporins (cephamycins) to β-lactamases suggested that a hydrogen atom at C-7 of the β-lactam ring (X in Fig. 1.4) might be replaced with advantage by a methoxyl group (OCH_3)[24,25].

The above expectations have been largely fulfilled by the development of two important series of broad spectrum cephalosporins. The first series represented by cephalexin 1.15 as the first orally-active cephalosporin to find wide clinical use. The second series is represented by cephalothin 1.16 and cephaloridine 1.17 as the first injectable cephalosporins effective against Gram-negative organisms [see Table 1.1, p.12].

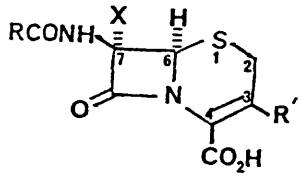
1.4 Biological properties of cephalosporins

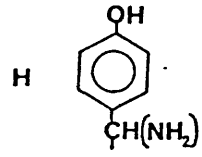
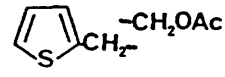
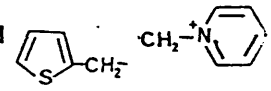
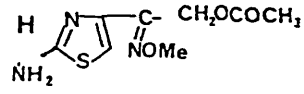
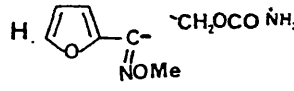
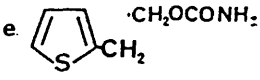
1.4.1 Pharmacological action and pharmacokinetic characteristics of cephalosporins

The cephalosporins are bactericidal and, similarly to the penicillins, they act by inhibiting synthesis of bacterial cell wall.

The first-generation cephalosporins (represented by cephalothin, Table 1.1, p.12) has good activity against a wide spectrum of Gram positive bacteria, and a modest one against Gram negative. As mentioned in section 1.3.3 chemical modifications of position 3 and 7 has resulted in a series of more potent cephalosporins. The second-generation compounds (represented by cefuroxime and cephamandole) have been characterised by their greater stability to hydrolysis by β-lactamases produced by Gram negative bacteria, while the third-generation (referred to as extended spectrum) cephalosporins, such as cefotaxime and cefsulodin, are even more stable to hydrolysis by β-lactamases than cefuroxime and cephamandole, with a wider spectrum and greater potency of activity against Gram negative

Table 1.1 : Some representatives of the a) orally and b) parenterally active semi-synthetic cephalosporins and their properties.



Trivial name	1st launch Company year country	X	R	R′	Activity and properties
(a) Orally active cephalosporins :					
Cephalexin 1.15	EliLilly 1969 USA Glaxo 1969 UK	H	$PhCH(NH_2)$	CH_3	Fair activity against G+ve, less activity against G-ve, very well absorbed and stable to β-lactamases.
Cefaclor	EliLilly 1979 UK	H	$PhCH(NH_2)$	Cl	More active than cephalexin against H.inluenza. Somewhat unstable chemically and in serum.
Cefadroxil	Bristol 1977 France	H		CH_3	Very similar to 15, but more sustained blood conc.
(b) Parenterally active cephalosporins :					
Cephalothin 1.16	EliLilly 1964 USA	H		$-CH_2OAc$	Active against G+ve and non-β-lactamase-producing G-ve organisms. Metabolically unstable.
Cephaloridine 1.17	Glaxo 1964 UK	H			Similar spectrum to cephalothin. Very stable in vivo
Cefotaxime	Hoechst, 1981 France Roussel	H		CH_2OCOCH_3	Very active against G-ve bacteria. Metabolically unstable.
Cefuroxime	Glaxo 1987 UK	H		CH_2OCONH_2	Active against broad range of G+ve & G-ve organisms Metabolically stable.
Cefoxitin	MSD 1978 USA	OMe		CH_2OCONH_2	Broad spectrum, active against B.fragilis, very stable to β-lactamases.

organisms[1].

Most cephalosporins can only be administered parenterally, as they are either inactivated by the stomach acid or not significantly absorbed from the intestine. Those agents that are absorbed from the gastrointestinal tract, such as cefadroxil and cephalexin, Table 1.1, are characterised by having electronegative substituents which improve their stability to gastric acid.

In general, cephalosporins administered orally in doses of 250 mg, 500 mg and 1 g produce peak plasma concentrations ranging from 6 to 30 μg/ml within two hours. Those given parenterally achieve average peak plasma concentrations of 40 μg/ml after 30 min of an intramuscular dose of 1 g[1].

Most cephalosporin antibiotics are 20 to 85 % bound to serum albumin as is fully discussed in Chapter 5. Serum protein binding inhibits drug passage across the capillary lining to tissues, the effect being greatest for cephalosporins having more than 85 % binding[26]. A high degree of binding may also prolong the presence of the drug in the blood, which partly explain the long serum half-life ($t_{1/2}$) of compounds such as ceftriaxone with a $t_{1/2}$ of 8.5 hrs. Generally, most cephalosporins are eliminated rapidly, with serum half-lives of 1 to 2 hrs, by the kidneys via glomerular filtration and active renal tubular secretion. In only a few cephalosporins is metabolism an important method of elimination. Hydrolysis of the β-lactam ring occurs within the body and produces inactive drug. Specific esterases metabolically remove 3-acetyl groups on the dihydrothiazine ring of compounds such as cefotaxime and cephalothin, which are then converted to the antibacterially inactive lactone of these substances[26,33].

1.4.2 Mechanism of action of cephalosporins

Bacteria are completely enclosed in a cell wall, which give them shape and form and protects them from harmful influences such as osmotic shock. Synthesis of bacterial cell

walls takes place in three distinct stages which occur at 3 different sites in the cell : the first stage involves synthesis of uridine nucleotide precursers, UDP-acetyl-muramyl-pentapeptide and UDP-acetyl-glucosamine, in the cytoplasmic region of the cell. The second stage is the utilization of the UDP, together with other substrates in the cell membrane, to introduce new disaccharide-pentapeptide units into the growing peptidoglycan of the cell wall, via a membrane-bound phospholipid. The third stage is the cross-linking of the linear peptidoglycan strands which takes place outside the cell membrane to form a peptide bridge, i.e. synthesis of the rigid component of the cell wall[27,28].

The action mechanism of the β-lactam antibiotics has been studied since the late 1940s. Strominger and Tipper[29] have shown that inhibition of bacteria by these compounds is likely to occur by reaction of penicillins and cephalosporins with transpeptidase enzymes important for the peptidoglycan cross-linking in the third stage of the cell wall synthesis (see Fig. 1.5). The bacterium is then unable to contain the high osmotic pressure of the cell contents, and the cell ultimately bursts.

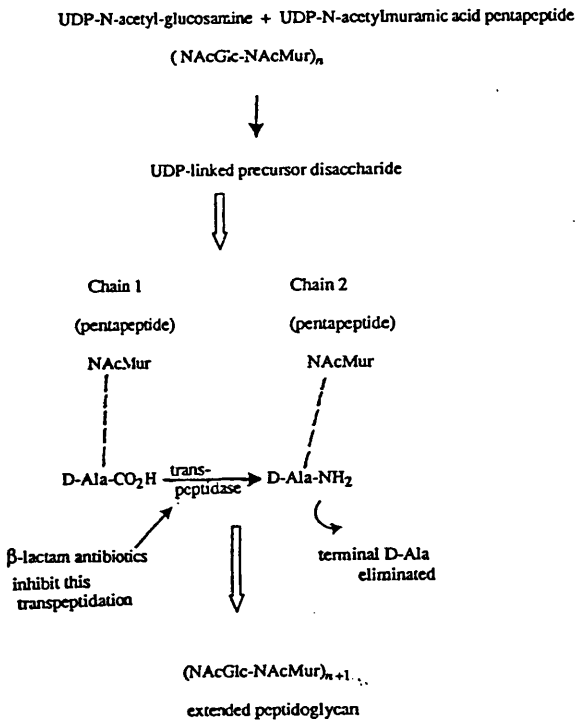


Fig.1.5

It has been suggested that the inhibition of the cross-linking enzymes by penicillin[30] and cephalosporins[31,32] involves a substrate analogue mechanism : the highly stressed amide group of the β-lactam ring is conformationally similar to the D-alanyl-D-alanine bond of the peptidoglycan pentapeptide. Consequently, the enzymes mistake β-lactam antibiotics for their normal substrates and react with them (irreversibly) to yield stable covalent esters which lack catalytic activity (see Fig. 1.6 below).

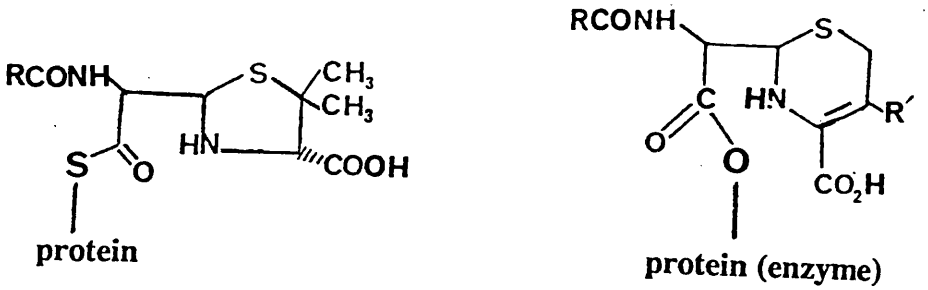


Fig.1.6

This irreversible acylation interferes with the synthesis of the cell wall in dividing cells, producing an osmotically unstable protoplast and eventually leading to the destruction of the cell. The exact molecular mechanism by which penicillins and cephalosporins inhibit cell wall synthesis in bacteria is not known[33]. In general, however, the dependence of the biological activity of an intact β-lactam function is rationalised by the above arguments. Van Heyningen and Ahern (1968)[34] and Morin and co-workers (1969)[7] have attempted to correlate activity with stability of the β-lactam of various cephalosporins. If the β-lactam is too stable, as in Δ^2-cephalosporins, the cephalosporin will presumably not acylate the transpeptidase and therefore will be biologically inactive. Such inactivity is indeed seen in the Δ^2-cephalosporins[34].

Note on side effects of cephalosporins

Cephalosporins generally cause few side effects. Hypersensitivity reactions are less common than with the penicillins and almost no cross-reactivity to cephalosporins have been reported in patients who have previously reacted to penicillins. Other hypersensitivity

reactions to cephalosporins include fever, arthralgia and exanthema. Some of the new cephalosporins have a 3-methylthiotetrazole side-chain, a moiety which confers a risk of reduced synthesis of prothrombin with subsequent risk of bleeding, and of disulfiram-like reaction in patients consuming alcohol following a cephalosporin dose[1,35].

1.5 Stability and degradation of β-lactam antibiotics

β-lactam antibiotics are the most widely used class of antimicrobial agents. Numerous penicillins and cephalosporins and their derivatives are now in regular clinical use, and many other new molecules belonging to this group of antibiotics are developed every year. Hence, stability and degradation studies related to the pure antibiotic compound should contribute to problems of their formulations and shelf-life, and data obtained are of great significance to pharmaceutical industry, also necessary for better understanding of these new agents.

In order to understand the factors affecting the antibacterial activity of β-lactam antibiotics, many studies have been done on the cleavage reaction of their β-lactam ring in alkaline aqueous solution and on those catalysed by β-lactamases with use of a variety of analytical methods such as iodometry[36], chromatography[36,37–39], UV spectroscopy[40–42] and microbiological assays[43].

Despite the resemblence in structure between the penicillins and cephalosporins (Fig. 1.7) there are striking differences in the products obtained on cleavage of their β-lactam rings[44a].

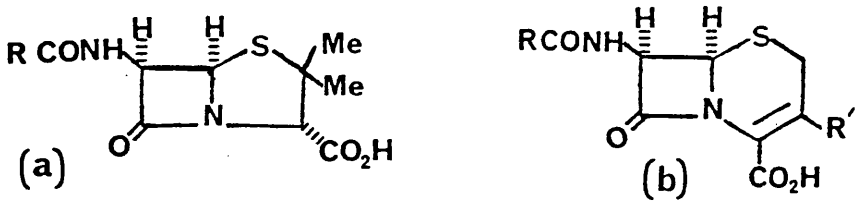


Fig. 1.7 : Structures of a) a penicillin and b) a cephalosporin.

Opening of the β-lactam ring of the penicillins by dilute alkali, penicillinase or reaction with amino groups yield mainly α-penicilloates (R,R). The penicilloate structure is relatively

stable, although epimerization may occur to give β (R,S), γ (S,R) and δ (S,S) diastereoisomers, which differ from the α compound in configuration at C-5 and/or C-6[41,45].

The presence of a Δ^3 double bond in the cephalosporin ring system as well as a substituent in position 3 leads to more extensive cleavage on hydrolytic degradation of the molecule, compounded by the fact that most of the degradation products are unstable.

A detailed account of the various mechanisms and sites involved in the degradation processes of β-lactam antibiotics is given in chapter four of this thesis, with more emphasis on those of cephalosporins which have been little studied in these respects.

Systematic studies on the degradation of cephalosporin derivatives are of interest for several reasons : a) a correlation between degradation and antibiotic activity has been shown in the first and second-generation cephalosporins[36], b) some degradation products may be involved in allergic reactions[46], and c) the stability of the compounds has to be known for the synthesis of derivatives[47] and the formulation of drugs.

The following is a brief review of the degradation of β-lactam antibiotics in various experimental conditions, as reported in the literature :

1.5.1 Effect of acid

The acidic degradation rates of penicillins are known to depend on the side-chain structure[48,49]. Benzylpenicillin is extremely acid unstable[50], while ampicillin is the most acid stable[51]. The side-chain reactivity of the penicillin molecule is attributed to the rearrangement initiated by the attack of the side chain amido carbonyl upon the β-lactam to produce the corresponding penicillenic and penillic acids[52].

Compared to the penicillins, cephalosporins are relatively insensitive to acid regardless of the N-acyl side chain because of the decreased nucleophilicity of the nitrogen in the dihydrothiazine ring[53]. β-lactams of these cephalosporins exhibit half-lives of about 25 hr at

pH 1.0 and 35° and are several times more stable than ampicillin under the same conditions[36]. However, in case of 3-acetoxymethylcephalosporins, such as cephalothin, the acetyl function is hydrolysed faster than the β-lactam moiety to yield the corresponding desacetyl compounds, which are easily converted to the lactones (see Scheme 4.9, chapter 4, p.120). The desacetoxycephalosporin derivatives, such as cephalexin and cephradine, were fairly acid stable even in strong acid solutions[36].

1.5.2 Effect of alkali

Some investigators stated that the enhanced sensitivity of alkaline degradation of cephalosporins and penicillins may be attributed mainly to a suppression of the usual amide resonance resulting from the nonplanarity in the β-lactam nitrogen atom[54,55]. Alkaline hydrolysis of cephalosporins results in cleavage of the β-lactam ring; however, the hydrolysis products formed seemingly do not correspond to those of penicillins, namely, penillic, penicilloic, and penicillenic acids. Some analogous compounds may have a transient existence during the hydrolysis[27]. The sensitivity of cephalosporins to degrade varied greatly with different 3-methylene substituents. In a series of 7-acylated cephalosporins, those with good leaving groups at position 3 were the most reactive; cephalexin and cephradine (with non-leaving groups at position 3) were the most resistant to the hydroxide-ion-catalysed degradation[36]. The significant influence of the substituents at the 3-methylene position upon the chemical reactivity of the β-lactam may be attributed to the long-range inductive effect on the electrophilicity of the β-lactam carbonyl carbon atom toward hydroxide-ion attack[56] and/or the leavability of the 3-methylene moiety, which may lower the energy of the transition state[57].

1.5.3 Enzymatic and aminolytic hydrolysis

Enzymology occupies a prominent place in the understanding of the mode of action of cephalosporins and penicillins and in appreciating some of the basis for bacterial resistance. β-Lactamases hydrolyse the β-lactam ring; the penicillins are converted to inactive

penicilloic acids; opening of the β-lactam ring of the cephalosporins is followed by more complex rearrangements. Of the three possible sites of enzymatic cleavage of cephalosporins, discussed by Pollock (1965)[58], the β-lactamase activity at site b (Fig. 1.8) has received far more attention than either the amidase (site a) or esterase activity (site c).

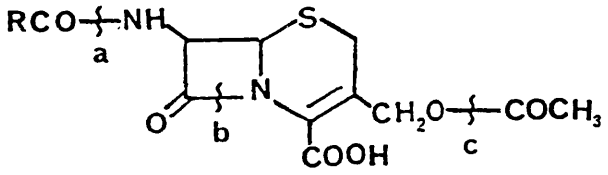


Fig.1.8 : Sites of enzymatic cleavage in cephalosporins.

Most observations during the β-lactamase decomposition and aminolysis of cephalosporins suggested that the initial product of the former involved hydrolysis of the β-lactam ring accompanied by elimination of the leaving group[59], while the product of the aminolysis involved only the hydrolysis of the β-lactam ring[60].

Table 1.2 (p.20) lists some of the analytical methods for the investigation of cephalosporin degradations, as reported in the literature.

Table 1.2[a] : Some of the analytical methods for the investigation of cephalosporin degradations as reported in literature :

Cephalosporin	Analytical method	Kinetics of degradation	evidence of degrad. products	Reference
Benzyl-penicillin	UV HPLC ^{1}H NMR	1st order epimerization & degradation	penicilloic, penicillenic & penillic acids	Clarke 1949[61] Blaha 1976[37] Degalaen 1979[62]
Cephazolin analogue	residual antib. det.iodometrically	pseudo 1st order pH 2-10	none	Rattie et al. 1979[63]
Cefadroxil	HPLC, pH profile colorimetric		2 piperazine diones isolated	Tsuji et al. 1981[64]
Cephalexin	UV	pseudo 1st order rates	piperazine -2, 5-dione	Indelicato et el. 1972, 74[65,66]
Cefaclor etc	Isolation of degrad. products		pyrazine dione + thiophene	Dinner 1977[67]
Various cephalosporins	UV, ^{1}H NMR	reactivity	^{1}H & ^{13}C NMR to follow degrad. mechanisms	Coene 1984[68] Sabath 1965[41]
Various cephalosporins	Polarography, iodometric, TLC		evidence of lactones etc.	Hall 1973[69]

Table 1.2[a] (conintued)

Cephalosporin	Analytical method	Kinetics of degradation	evidence of degrad. products	Reference
3-acetoxy cephalosporins	HPLC	entire pH range	evidence of lactones etc.	Yamana et al. (1976)[36]
Cefixime	HPLC	Rate constant	much evidence	Namiki 1987[70]
Various cephalosporins	UV (decrease at A_{250nm})	enzymic hydrolysis	None	Sabath et al. 1965[41]
Cephaloridine	UV (decrease in A_{260})	β-lactamase reaction	None	Faraci et al. (1984)[59]
Cephacetrile	HPLC	Assay	desacetyl (use enzyme), lactone	Mangia et al. (1979)[71]
Cephalothin			desacetyl and lactone	Indelicato et al. (1985)[72]
Various cephalosporins	UV (decrease in A_{260})	aminolysis & alk. hydrolysis	used standard compounds	Bundgaard[73] (1975)
Various cephalosporins	UV/ ^{1}H NMR	aminolysis & alk. hydrolysis	used standard compounds	Hamilton-Millor (1970)[44a,b]

Table 1.2[a] (conintued)

Cephalosporin	Analytical method	Kinetics of degradation	evidence of degrad. products	Referenc
Cephalexin	UV, HPLC	rate constant pH4-7	None, no hard evidence	Das Gupta (1981)[40]
Cephradine	^{1}H NMR	Alkaline degradation	piperazinedione isolated	Cohen 1973[74] Indelicato 1972[65]
Cefotaxime	HPLC, electro-chemical detection		use of standard lactone	Fabre 1985[75] Fabre et al. 1986[76]
Cefamandole	constant pH titration, NMR	pseudo 1st order rate constant	conversion of nafate to free alcohol	Indelicato[77] 1976
	HPLC (nafate)			Fabre 1982[78]
	HPLC			Das Gupta 1981[40]
	Microbiol., TLC			Borstein 1980[79]
	Polarog. etc			
Moxalactam	HPLC	epimerization		Hashimoto 1985[81]
	pH titration	mechanisms		
	pH titration	epimer. & degrad.		Hashimoto 1984[80]
		epimer.in frozen soln.		Hashimoto 1988[82]

a antib.=antibiotic, det.=determined, degrad.=degradation, alk.=alkaline, Microbiol.=Microbiological, Polarog.=Polarography, epimer.=epimerization.

Aims and objects of the present work

This introduction has brought to light several aspects of the cephalosporin antibiotics which require further investigation.

The sources of materials used in this work are described in Chapter Two. This section includes a variety of synthetic procedures to produce reference standards and related compounds.

The abundance of cephalosporin antibiotics (and of β-lactam antibiotics in general) in present day clinical use presents a major challenge to pharmaceutical analysts both from the view points of specific identification and quantification, and detection of impurities and products of degradation and isomerization. The value of NMR spectroscopy in differentiating groups of closely related compounds is well known and of direct application to cephalosporin derivatives which form a group of this kind. Some work of this nature has already been carried out at Bath[83,84] but as a result of the recent introduction of several novel cephalosporin antibiotics, an extension of such NMR investigations has been undertaken as part of this Thesis (Chapter 3).

The value of NMR spectroscopy in studies of the stability and degradation of β-lactam antibiotics of the penicillin class has already been demonstrated[44,62,85] and it appeared logical to extend its application to the same problem in regard to the cephalosporins. This aspect forms a second theme of this Thesis (Chapter 4).

Finally the question of the binding of cephalosporin antibiotics to serum albumin has been taken up on account of the key role it plays in determining the pharmacokinetics of β-lactam antibiotics. Binding studies reported in the literature mostly employ classical techniques, and that of spectrofluorimetry has been employed in the present work by use of state-of-the-art instrumentation. However, evidence of binding parameters may also be gained from NMR relaxation times, and this alternative technique has also been investigated

in regard to cephalosporins (Chapter 5 of this Thesis).

CHAPTER TWO

Source of materials

2.1 Drug samples obtained from the manufacturers

Table 2.1 below lists the drug samples employed in the present investigation and their manufacturers. Details of their chemical structures are given in Table 2.2 (p.33). The compounds are listed alphabetically by generic name in Table 2.2 to enable easy reference to structure throughout the Thesis and to avoid undue duplication of formulae.

Table 2.1 :

Drug name	Batch No.	Manufacturer
Benzylpenicillin Na	3DP 8578A	Glaxo, England
7-ADCA	UCC 2749	Glaxo, England
Cephalexin	KT/17	Glaxo, England
Cephradine	5M80710	Squibb, USA
Cefadroxil	CCD8UO914	Bristol Sermoneta, Latina,Italy
Cefaclor	82K774	Eli Lilly, England
Cephalothin Na	FFOFO4A	Eli Lilly, England
Cephaloridine Na (Ceporin)	4033	Glaxo, England

Cefoxitin Na (Mefoxin)	18423	MSD, Hertfordshire
Cefuroxime Na (Zinacef)	8CP 114B	Glaxo, England
Cefotaxime Na (Claforan)	0045	Roussel, England
Ceftizoxime Na (Cefizox)	E9149A	Wellcome Foundation, Dartford
Ceftriaxone Na (Rocephin)	707	Roche, Hertfordshire
Cefixime Na	RM 27	Cyanamid, USA
Ceftazidime pentahydrate (Fortum)	B6166LE	Glaxo, England
Cefsulodin Na (Monospor)	85-192591	Ciba-Geigy, Horsham
Cephamandole Li	G95-39M	Eli Lilly, England
Cephamandole nafate	FF2JO5C	Eli Lilly, England
Cephazolin Na	5SJ36	Eli Lilly, England
Cefatrizine	20410	Bristol laboratories, Syracuse, USA
Moxalactam $2NH_4$ (1-O-analogue)	C34-2W6-84	EliLilly, USA
Cefotaxime lactone	8A0291B	Roussel UCLAF
Cefotaxime free acid	8A4124B	Roussel UCLAF
Desacetylcefotaxime	8A0203B	Roussel UCLAF

2.2 Synthesis of some cephalosporin degradation products and related compounds

2.2.1 Introduction

The following compounds were synthesised by standard methods or modifications thereof. The original reference is given in the relevent section. The other compounds used in the present work were either obtained from the original researchers or from commercial companies.

2.2.2 Experimental

Melting points were determined on a Gallenkamp electrically heated melting point apparatus. Kieselgel 60 F_{254} (silica gel) plates were used for thin-layer chromatography (TLC) and were visualised using an iodine vapour chamber. Solvent evaporation under reduced pressure was carried out using a Buchi Rotavapour R110 rotary evaporator. Lyophilization was carried out using an Edwards Modulyo freeze-drier operating at ~ -40°/3 mbar.

^{1}H and ^{13}C NMR were recorded on a Jeol JNM-GX-270 spectrometer (full operating details are given in Chapter three, p.38). Unless otherwise stated assignments were established by comparison with published data and/or on the basis of expected chemical shift, signal multiplicity and the use of DEPT ^{13}C NMR pulse sequences.

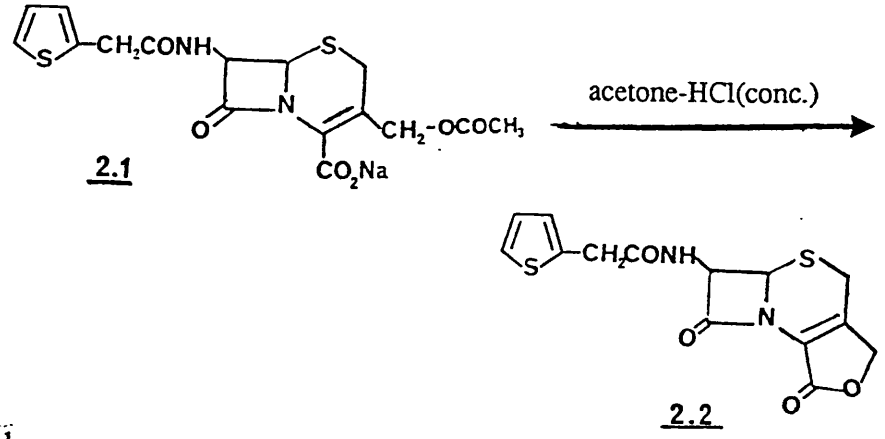
2.2.3 Compounds prepared

2.2.3.1 Desacetylcephalothin lactone 2.2

The compound was synthesised according to the method of Neidleman et al.[86], as follows:

To a solution of 550 mg of cephalothin Na 2.1 in 6 ml of distilled water were added 5 ml of acetone and 2 ml of concentrated hydrochloric acid (HCl, pH 1.4). The reaction mixture was stirred at room temperature (24°C) overnight. The formation of the lactone, followed by

TLC (EtOH : $CHCl_3$: CH_3COOH, 50 : 100 : 7.5), took place 1/2 hour after acidification in the form of a white precipitate. The total yield of desacetylcephalothin lactone 2.2 was 338 mg (62%), collected by filtration, washed with water, and dried in vacuum desiccator. The material 2.2 was further purified to remove residual traces of 2.1, as follows : to the crude product 2.2 in 75 ml of acetone/ ethyl acetate (1 : 2) was added 10 ml of 5% aqueous $NaHCO_3$, followed by 10 ml of water. The mixture was shaken vigorously, then the phases were allowed to separate (for at least 2 hours). The upper layer, containing the lactone 2.2 completely free of residual unreacted material, was concentrated in vacuo on a rotary evaporator at room temperature. Crystalline 2.2 separated, m.pt. 225 - 228°C (with decomposition); literature (Neidleman et al., 1970[86]) : 228 - 230°C (with decomposition).



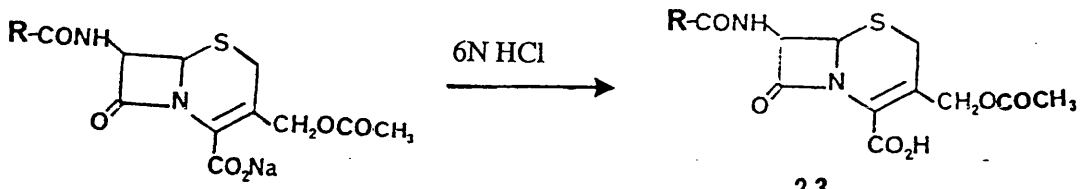
2.1 2.2

The ^1H NMR spectral data are consistent with structure 2.2 (in DMSO-d_6) : 3.49 ppm (s, 2-CH_2), 3.87, 3.93 ppm (dd, ArCH_2), 5.17 ppm (brs, lactone CH_2), 5.20 ppm (d, J=5 Hz, 6-H), 5.97 ppm (dd, J=5 & 8.4 Hz, 7-H), 7.07 ppm (m, 2H, thienyl), 7.49 ppm (m, 1H, thienyl), 9.33 ppm (d, J=8.4 Hz, NH exchangeable), (see also Table 3.3, p.64).

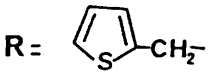
2.2.3.2 Preparation of cephalothin free acid 2.3[87]

A solution of the sodium salt of cephalothin 2.1 (1.0 g) in 30 ml of distilled water was chilled in an ice bath with constant stirring. The pH was adjusted to 1.8 - 2.0 by dropwise addition of 6N HCl. The precipitated cephalothin free acid 2.3 was then filtered and washed twice with cold water (5 ml each). The product was stored under vacuum overnight. The

yield was ~ 85%.



2.1 2.3



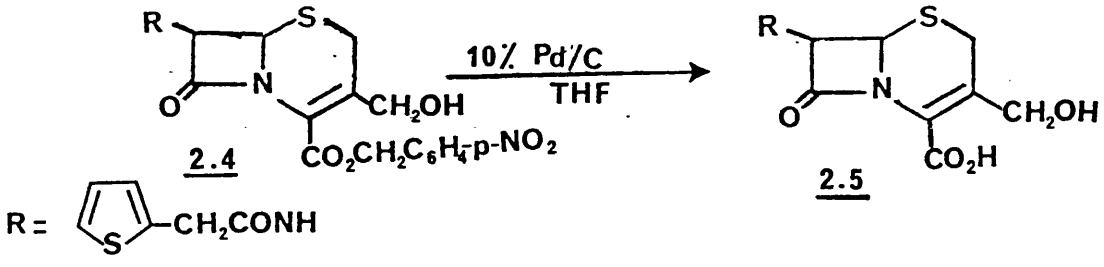
The ^{1}H NMR data are consistent with structure 2.3 (ppm, DMSO-d_6) : 2.03 ppm (s, OCOMe), 3.49, 3.63 ppm (dd, J=18 Hz, 2-CH_2), 3.80 ppm (dd, J=14.5 Hz, ArCH_2), 4.90 ppm (dd, J=13 Hz, 3'-CH_2), 5.10 ppm (d, J=4.8 Hz, 6-H), 5.71 ppm (dd, J=4.8 Hz, 7-H), 6.94 ppm (m, 2H, thienyl), 7.21 ppm (m, 1H, thienyl), 9.1 ppm (d, J=8.1 Hz, NH, exchangeable).

2.2.3.3 Preparation of desacetylcephalothin 2.5

2.5 was obtained from the 4-p-nitrobenzyl ester of desacetylcephalothin 2.4 (kindly supplied by Dr. M. Johnson, University of Chicago) by catalytic hydrogenolysis for the removal of the p-nitrobenzyl group, according to the method of Kukolja et al.[88], as follows :

A solution of 500 mg of the p-nitrobenzyl ester 2.4 in 30 ml tetrahydrofuran (THF) and 15 ml methanol containing 1 ml of 1N HCl and 500 mg of 10% palladium on carbon was stirred at 25°C for 2 hours under 60 psi H_2. The reaction mixture was filtered, and washed successively with 10 ml THF, 5 ml MeOH, and 5 ml ethyl acetate. The filtrates were combined and evaporated to dryness in vacuo. The residue was redissolved in 10 ml ethyl acetate-water (1 : 1), and the pH was adjusted to 7.0 with 1N NaOH. The aqueous phase was separated, washed with ethyl acetate, and lyophilized to yield the sodium salt of 2.5. Otherwise, to obtain the free acid 2.5, the organic layer was removed and discarded (containing the p-nitrobenzyl polymer). The aqueous layer was extracted once more with ethyl acetate, acidified to pH 4.25 by addition of 1N HCl, and lyophilized. The powder was

suspended in 5 ml water, the pH adjusted to 4.25, and the mixture filtered to give 55% of unpurified 2.5.



^{1}H NMR (ppm, DMSO-d_6) : 3.31, 3.48 ppm (dd, J=18 Hz, 2-CH_2), 3.86, 4.18 ppm (dd, J=12.4 Hz, 3'-CH_2), 4.9 ppm (d, J=4.9 Hz, 6-H), 5.48 ppm (dd, J=4.9 and 8.4 Hz, 7-H), 6.9 ppm (m, 2H, thienyl), 7.4 ppm (m, 1H, thienyl), 9.0 ppm (d, J=8.4 Hz, NH, exchangeable). (See also Table 4.7, p.173).

2.2.3.4 Preparation of p-nitrobenzyl ester of cephalothin 2.6

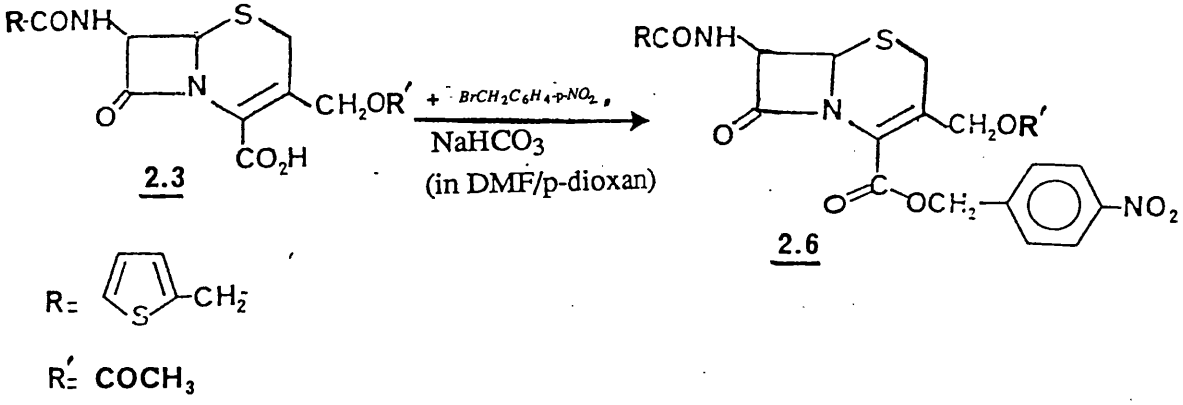
The ester 2.6 was obtained from cephalothin (free acid) 2.3 according to the method of Mobashery et al.(1986)[87], as follows :

A suspension of cephalothin (450 mg, 1.14 mmol), p-nitrobenzyl bromide (300 mg, 1.39 mmol), $NaHCO_3$ (110 mg, 1.25 mmol), in a 5 : 4 mixture of dimethylformamide (DMF) and p-dioxan (3 ml) was stirred at room temperature (~ 24°C) overnight. The solution was then poured into a mixture of saturated $CaCl_2$ and ethyl acetate (10 ml). The organic layer was subsequently washed with 2 ml saturated $CaCl_2$, 3 ml water, 2 ml saturated $NaHCO_3$, and 2 ml water. After drying the ethyl acetate layer over anhydrous magnesium sulphate, it was evaporated to dryness in a Buchi rotavaporizer. The resulting residue was crystallized from ethyl acetate at -20°C to afford a yield of 45 % of a pale yellow solid. TLC, 5 : 4 : 0.5 : 0.5 toluene-p-dioxan-EtOH absolute- dilute ammonia solution, one spot R_f=0.49, m.pt. 138-140°C, (literature[87], 147-148°C).

Elemental micro-analysis : Found C, 51.80%; H, 3.94%; N, 7.87%.

$C_{23}H_{21}N_3O_8S_2$ requires : C, 51.97%; H, 3.98%; N, 7.90%.

See Table 3.2 (Chapter 3, p.63) for ^1H NMR data, and Table 3.15 (Chapter 3, p.92) for ^{13}C NMR data.

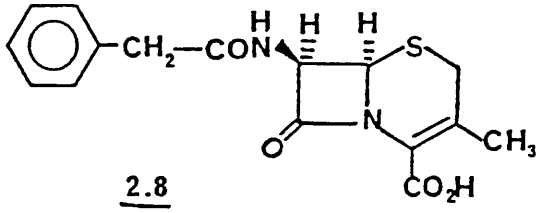


2.2.3.5 7-(2-phenylacetamido)desacetoxy-Δ^3-cephalosporanic acid 2.8

A sample of the p-nitrobenzyl ester of 7-(2-phenylacetamido)desacetoxy Δ^3-cephalosporanic acid 2.7 was kindly supplied by Glaxo Laboratories, Greenford. The protective ester function was removed according to the method of Kukolja et al. (1985)[88], as follows :

A solution of 5.2 g of the p-nitrobenzyl ester of 7-(2-phenylacetamido desacetoxycephalosporanic acid 2.7 in 150 ml of methanol containing 10 ml of 1N HCl and 5.2 g of 10% palladium on carbon was stirred at 25°C for 2 hours under 60 psi H_2. The reaction mixture was filtered and the filtrate was concentrated to give a gum. The gum was dissolved in 40 ml of water and 40 ml ethyl acetate. The mixture was neutralised to pH 7.0 by addition of 1N NaOH, and the organic layer was removed and discarded. The aqueous layer was either washed once more with ethyl acetate (to remove the p-nitrobenzyl polymer) and then lyophilized to yield the sodium salt of 2.8, or acidified to pH 4.25 by addition of 1N HCl. The aqueous acid solution was lyophilized to afford ~ 1.62 g (30%) of 2.8 as the free acid. M.pt. 186-188°C (with decomposition), (no literature m.pt.)

The ^{1}H (Table 3.3, p.64) and ^{13}C NMR data (Table 3.5,p.76) were consistent with structure 2.8.



2.8

2.2.3.6 Synthesis of ceph-2-ene 2.9

7-phenylacetamido-3-methyl-2-cephem-4-carboxylate 2.9 was synthesised in our laboratories according to the method of Lammert et al.[89], as follows :

A mixture of 0.483g (0.001M) of 4-p-nitrobenzyl-7-phenylacetamido-3-methyl-Δ^3-cephalosporin 2.7, 10 ml tetrahydrofuran (THF), 10 ml of water, and 0.24 g (0.001M) of sodium sulphide nonahydrate ($Na_2S.9H_2O$, mol.wt 240) was stirred in an ice-bath for 30 minutes. HCl (1 ml, 1N) was added and the THF was evaporated on a rotavaporizer and the solution then extracted with 10 ml ethyl acetate. The extract was discarded. The pH of the aqueous layer was adjusted to 3.2 with hydrochloric acid, and the oily product commenced to crystallize. The crystalline acid 2.9 was then collected by filtration, washed with ethyl acetate and dried in a vacuum oven overnight.

Yield, 0.314 g (65%); m.pt. 170-173°C . (literature : 167-169)[89]. (See Tables 3.17 (p.99) and Table 3.19 (p.101) for ^{1}H NMR and ^{13}C NMR features, respectively).

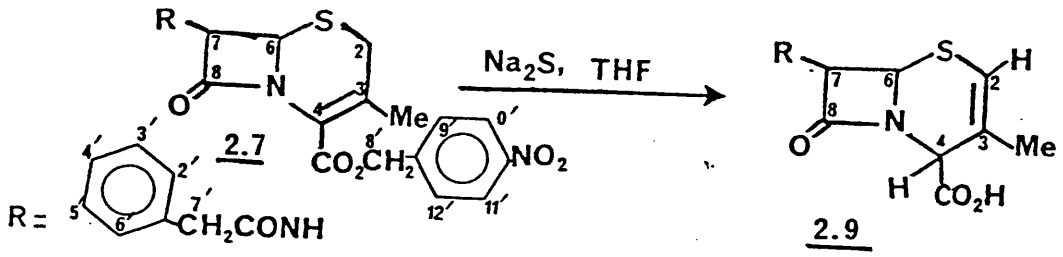
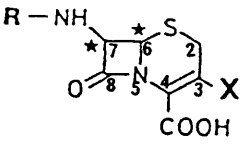


Table 2.2 :

Cephalosporins of general formula :



(alphabetically listed by generic name, as listed in the 29th Ed. of Martindale[1])

(chiral centres starred)

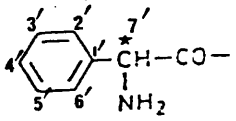
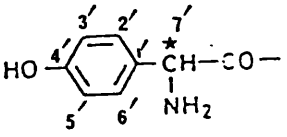
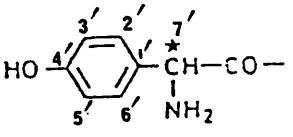
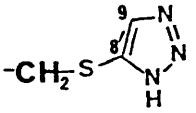
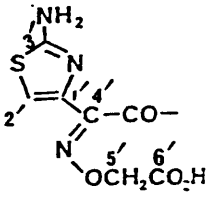
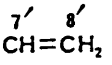
Generic name	R	X
7-ADCA	**H**	**$^{3'}CH_3$**
cefaclor		**Cl**
cefadroxil		**CH_3**
cefatrizine		
cefixime		

Table 2.2 (continued)

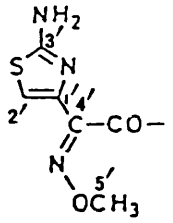
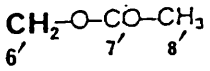
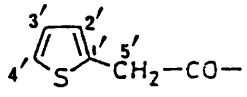
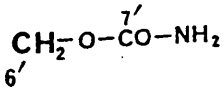
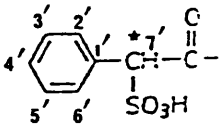
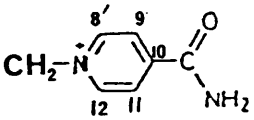
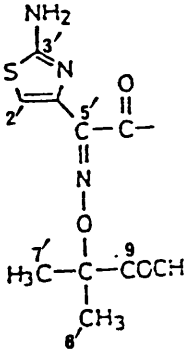
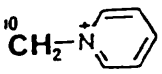
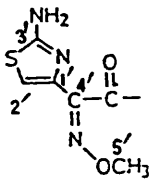
Generic name	R	X
cefotaxime		
cefoxitin (cefamycin example with 7-OCH_3 substituent)		
cefsulodin		
ceftazidine		
ceftizoxime		H

Table 2.2 (continued)

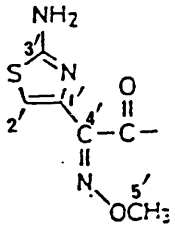
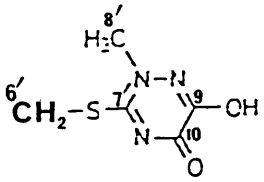
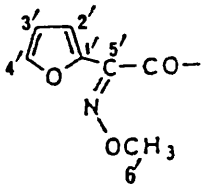
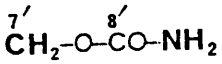
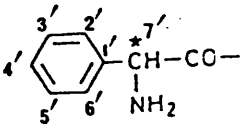
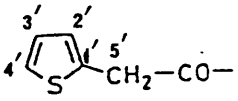
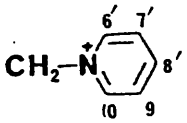
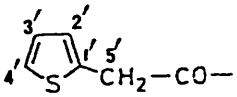
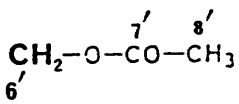
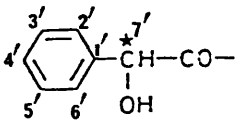
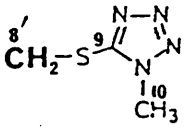
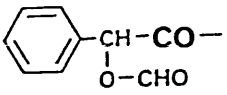
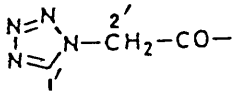
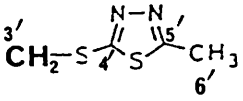
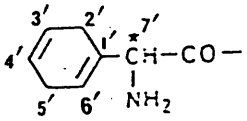
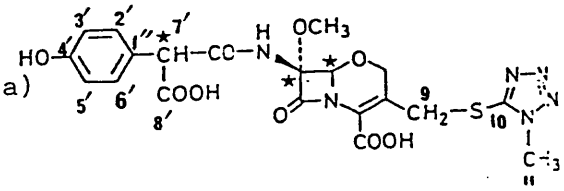
Generic name	R	X
ceftriaxone		
cefuroxime		
cephalexin		CH_3
cephaloridine		
cephalothin		
cephamandole		

Table 2.2 (continued)

Generic name	R	X
cephamandole nafate		as above
cephazolin		
cephradine		CH_3
moxalactam (complete formula)		

CHAPTER THREE

High-field proton (270 & 400 MHz) and carbon-13 (67.8 MHz) NMR analysis of some cephalosporins and their derivatives

3.1 Aim

General spectral features of some cephlosporins and their derivatives, as well as specific resonances, are given and discussed and their application to the identification of these antibiotics is outlined.

3.2 Introduction

NMR spectroscopy combines the advantages of high specificity with rapid analysis and minimal sample preparation. Numerous reports on the ^{1}H and ^{13}C NMR spectra of cephalosporins have appeared in the literature [90–94] . Many of these deal with spectral assignments of derivatives in organic solvents such as deuterochloroform, while others use NMR spectroscopy to determine the structure of underivatised cephalosporins. The ^{1}H and ^{13}C NMR data for a variety of penicillin and cephalosporin antibiotics have recently been reviewed[83,84].

In this work, high-field ^{1}H (270 and 400 MHz) and ^{13}C (67.8 MHz) NMR data, obtained in our own laboratories, under standard conditions, of some cephalosporins and their derivatives are presented. Some of our data are novel, run at high-field to clarify features of lower resolution spectra and to provide information on isomeric nature where appropriate. The work includes confirmation of the structure of esters obtained from industry and those prepared in our own laboratories, and also of lactones and some reference compounds of potential value to degradation studies.

Spin-lattice relaxation time (T_1) measurements have been utilised for the identification and discrimination of some quaternary carbon atoms.

3.3 Experimental

3.3.1 Instrumentation

The natural-abundance ^{13}C NMR spectra were recorded at 67.8 MHz on a Jeol JNM-GX-270 Fourier Transform (F.T.) spectrometer. Proton noise-decoupled spectra with NOE (Nuclear Overhauser Enhancement) were recorded under the following conditions: ambient temperature (20°C), spectral width 18050 Hz (=266 ppm), 16K data points (zero filled to 32 K), a 4.0 μs pulse with a 0.454 sec. acquisition time and a 0.213 sec pulse delay. The normal proton noise-decoupled spectra were supplemented by DEPT[95] (Distortionless Enhancement by Polarization Transfer) experiments set to measure CH, CH_3(positive) and CH_2(inverted) (pulse angle= 135°), or CH alone (pulse angle= 90°); C_q resonances are absent in 135° and 90° DEPT.

The 270 MHz ^1H FT NMR spectra were recorded on the Jeol JNM-GX-270 instrument, operating at an ambient temperature, using a 32 K data points and a frequency of 3001.2 Hz giving a digital resolution of 0.18 Hz. A 5.0 μs pulse corresponding to a tilt angle of 30°C was employed with a 5.459 sec acquisition time and a 0.541 s pulse delay between pulses. The ^1H-^1H 2D correlated spectroscopy (COSY) experiments on ceph-2-enes were performed by Dr. Sarah Branch, Department of Pharmaceutical Chemistry, University of Bath.

The 400 MHz ^1H FT NMR spectra were recorded on a Jeol-GX400 spectrometer, at Department of Chemistry, University of Bristol (range -0.4 to 9.6 ppm). A sweep width of 4000 Hz was employed with 32 K data points, thus giving a digital resolution of 0.24Hz. The pulse width was 0.6 μs and the repetition rate was 5.277 sec (acquisition time plus 2.0 sec pulse delay).

The inversion-recovery method[96] was used for the spin-lattice relaxation time (T_1) measurements for carbons 3 and 4. The carbon-13 spectra were recorded on Jeol-GX270 spectrometer with 10 different τ values ranging from 0.05-10 sec, under the following

conditions : ambient temperature (20°C), spectral width 18050 Hz, 16 K data points, a 21 μs pulse (PW1) corresponding to an angle of 90° and a 42 μs pulse (PW2) corresponding to a 180° angle and a 10 sec pulse delay between pulses.

3.3.2 Materials and methods

Cephalosporin antibiotics samples were provided by various pharmaceutical companies (details in Chapter 2, Table 2.1). They were generally of a purity suitable for pharmaceutical formulations. Some of the cephalosporin esters, lactones and other derivatives were synthesised in our laboratories (see Chapter 2, section 2.2).

Samples were prepared in 5 mm o.d. tubes as 5-10 %w/v solutions in D_2O, DMSO-d_6, $CDCl_3$ and other deuterated solvents. Tetramethylsilane (TMS) and sodium 2,2-dimethyl-2-silapentane-5- sulphonate (DSS) were used as chemical shift standards, δ=0.00 ppm. In most experiments with D_2O as solvent the large singlet at δ_H=4.80 ppm due to HOD was used as a reference (DSS complicates the higher field region of the spectrum).

Alkali metal salts of cephalosporins were freely soluble in D_2O while the solution of free acids was promoted by minimal quantities of alkali (usually $NaHCO_3$), or acid (triflouroaceticacid, TFA) in the case of β-lactams with amino substituents such as cephalexin. The cephalosporin esters were examined in $CDCl_3$ or DMSO-d_6.

Assignments of proton and carbon-13 signals were based on the expected chemical shift considerations and signal multiplicities, and on correlations with published data and reference spectra.

Carbon-13 assignments were aided by the use of DEPT pulse sequences, allowing the distinction between the various orders of carbon protonation. In addition, some ^{13}C-^{1}H coupled spectra were run to aid the assignments of complicated spectra. Also ^{1}H- ^{1}H 2D COSY experiments were employed to aid the confirmation of ceph-2-ene configuration at

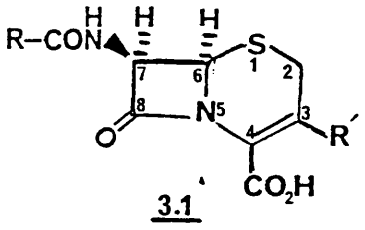
C-4. DEPT and COSY techniques have been recently reviewed[95].

Chemical shift (δ ppm) data for each cephalosporin or related compounds in the appropriate solvent, as well as the multiplet separations (first-order J values), were obtained from the instrumental print-outs.

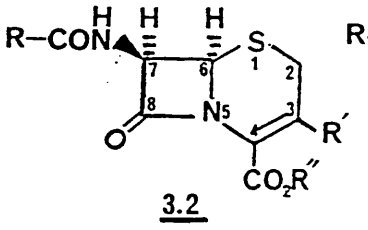
3.4 Results and discussion

The ^{1}H NMR data of cephalosporins, cephalosporin esters, and other related compounds are listed in Tables 3.1 (p.51-54), 3.2 (p.63) and 3.3 (p.64), respectively. The ^{13}C NMR spectral characteristics of the above are listed in Tables 3.4 (p.74-75), 3.5 (p.76), those of the esters were discussed in detail in section 3.4.8 (p.**88**). These data include cephalosporins recently introduced into clinical practice.

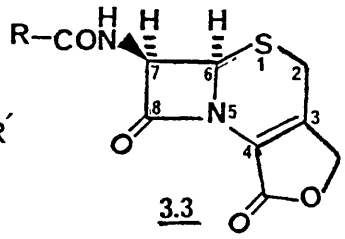
The general structure, ring numbering and absolute stereochemistry of the free acid form of the antibiotic is given in formula 3.1; the structures of the esters and lactones are given in formulae 3.2 and 3.3, respectively; details of substituents and generic names are included in the Tables for easy reference.



3.1



3.2



3.3

Cephlosporin (cephem nucleus) 6-R, 7-R	cephalosporin ester 6-R, 7-R	cephalosporin lactone 6-R, 7-R

Tables 3.1-3.3 summarise recorded ^{1}H NMR data for a variety of cephalosporin derivatives, while Fig. 3.1 (p.41) illustrates a typical ^{1}H spectrum obtained for cephalothin as one of the most common cephalosporin antibiotics.

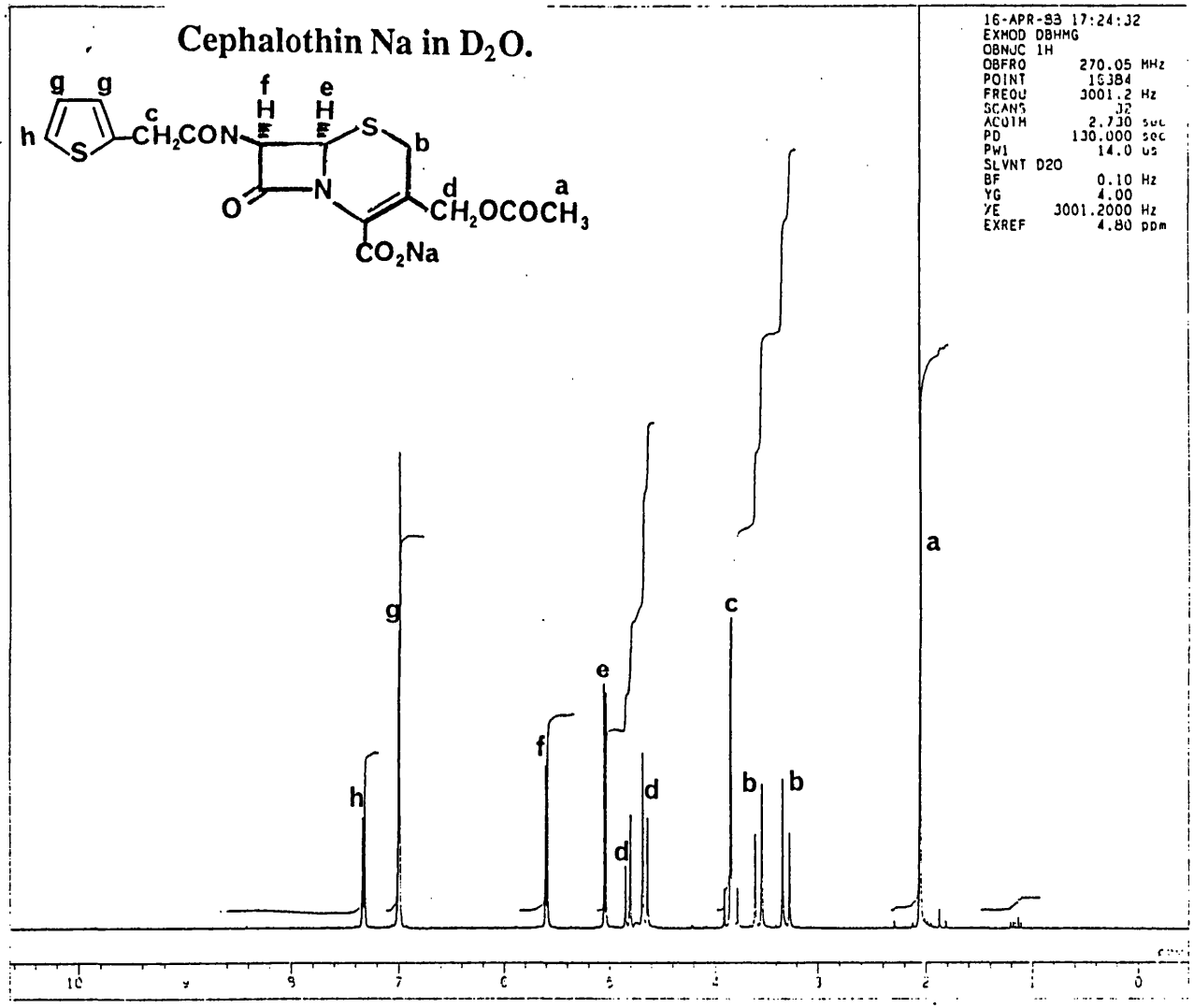


Fig. 3.1 : ^1H NMR spectrum of cephalothin Na, recorded at 270 MHz in D_2O to illustrate characteristic spectral features of cephalosporin antibiotics. (Experimental conditions as in section 3.3.1)

3.4.1 ^{1}H NMR features of cephalosporins

From the above mentioned Tables and Figure the ^{1}H NMR spectral characteristics of cephalosporin derivatives can be classified into : a) common and b) specific spectral features.

3.4.1.1 Common ^{1}H NMR spectral features

(i.e.,common to all examples)

1. β-lactam ring 6-H and 7-H proton resonances

Most characteristic of the ^{1}H NMR spectra of all cephalosporins, having an intact β-lactam ring, in D_2O are the two single-proton doublets centred at 4.85 - 5.35 ppm and 5.50 - 5.95 ppm for the 6-H and 7-H, respectively, in the β-lactam ring, forming an AB doublet pair, with ^3J coupling values of 4-5 Hz (Table 3.1) characteristic of cis protons in lactams[97, 98]. Furthermore, the cephalosporins β-lactam protons are characterised by their large chemical shift separations ($\Delta_{6,7}$=0.5-0.9 ppm) compared to those of the penicillin antibiotics (e.g.,benzylpenicillin, $\Delta_{5,6}$=0.06 ppm). Green et al.[93], reported that this feature is distinctive enough to enable differentiation of cephalosporins from penicillins.

The ^{1}H NMR spectra of the free acids, lactones and esters of cephalosporins in deuterochloroform ($CDCl_3$) or deuterated dimethylsulphoxide (DMSO-d_6) are characterised by a single-proton double doublet (dd) centred at 5.60-6.00 ppm and a single-proton doublet centred at 4.75-5.15 ppm for the 7-H and 6-H, respectively, in the β-lactam ring. The 7-H double doublet signal is attributed to coupling with 6-H on the one side (J=4.5-5.2 Hz) and with the amido proton on the other (J=8.0-9.3 Hz). This assignment is confirmed by deuteration (addition of D_2O), when the dd collapses to a doublet with the same coupling constant (4-5 Hz) as that shown by the 6-H doublet; the amido proton doublet at 6.10 - 6.40 ppm (in $CDCl_3$) and 9.1 - 9.6 ppm (in DMSO-d_6) disappears. This is illustrated by the spectra of cefotaxime Na in DMSO-d_6 (Fig. 3.2a, p.43) before and after addition of a drop of D_2O and shaking (Fig. 3.2b). In the latter case the amido proton in D_2O undergoes exchange

with deuterium so that only the doublets for the 6- and 7-H appear.

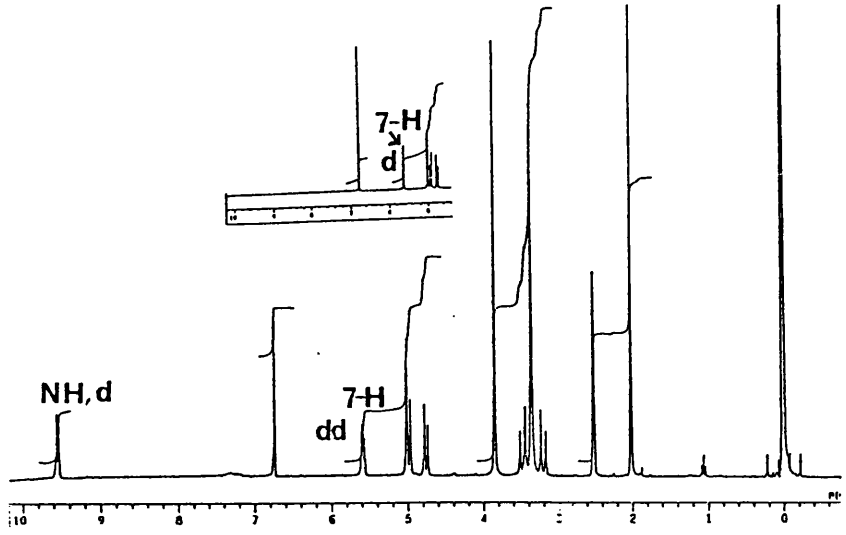


Fig. 3.2 : 270 MHz ^{1}H NMR spectrum of cefotaxime Na in DMSO-d_6. Insert : partial spectrum after addition of D_2O and shaking to illustrate the collapse of the 7-H double doublet to a doublet. (Experimental conditions as in section 3.3.1).

In cephalosporins with a methoxy group replacing the usual proton at position-7 in the β-lactam ring (e.g. 7-OMe of cefoxitin and moxalactam) the 6-H resonates as a singlet at 5.10 - 5.12 ppm because there is no 7-H with which it can couple to form the characteristic doublet of the AB system.

2. Thiazinyl ring protons 2-CH_2 resonances

The ^{1}H NMR spectra of cephalosporin derivatives are further characterised by the two-proton AB doublet pairs, due to the methylene group at position-2 of the dihydrothiazine ring, having the two protons in different chemical environments. They are distinguished from the lactam resonances by their higher chemical shifts (range 3.0-3.9 ppm) and characteristically large separations (^2J ~ 18 Hz), typical of saturated geminal methylene protons in which the coupling range is given as -20 to +40 Hz [99] ; similar features are also shown by esters and free acids [Tables 3.2 and 3.3]. Although the 2-CH_2 signal appeared as two well resolved doublets, with the inner lines more intense than the outer (Fig. 3.1, p.41),

their chemical shift separations (0.10-0.35 ppm) are much smaller compared to those of β-lactam protons($\Delta_{6,7}$ 0.5-0.9 ppm). Generally, the chemical shift of the 2-CH_2 protons reported above is relatively low for methylene protons. This is due to the deshielding effect of the adjacent sulphur atom and olefinic group. The 2-CH_2 resonances are lower field in 1-oxo-analogues, such as moxalactam (centred at 4.36 and 4.53 ppm, J=17.2 Hz, Table 3.1), due to the greater deshielding influence of oxygen compared with that of sulphur.

3.4.1.2 Specific ^{1}H NMR spectral features

1. Signals to higher field of 6.0 ppm

a) 3-proton (methyl) singlets

Certain 3-proton singlets due to methyl attached to carbon in the range 2.0 - 3.0 ppm, nitrogen or oxygen in the range 3.5 - 4.0 ppm are valuable aids to identification. In compounds having more than one methyl group in their structure, care is required to differentiate them (this is not always easy). Table 3.7 below summarises the chemical shift ranges of the various types of the methyl groups of cephalosporins.

Table 3.7 : Summary of the chemical shift ranges of various types of methyl groups of cephalosporins :

Me type	Examples	δ range in ppm
3-Me	cephalexin, cefadroxil	1.90 - 2.15 s
-OCOMe	cephalothin, cefotaxime	2.05 - 2.10 s
Ar-Me	cephazolin	2.70 - 2.75 s
7-OMe	cefoxitin, moxalactam	3.40 - 3.51 s
NMe	cephamandole, ceftriaxone	3.55 - 4.00 s
NOMe	cefuroxime, ceftizoxime	3.90 - 4.00 s

s = singlet, Ar = Aromatic.

b) CH_2 of 3-CH_2X type derivatives

Most of the cephalosporin derivatives are of this type which add an additional 4-line AB signal, causing further complexities to cephalosporin spectra. This signal falls between the 6-H, 7-H (4.9 - 6.0 ppm) and 2-CH_2 (3.0 - 3.9 ppm) resonances, having chemical shift range of 3.95 - 5.65 ppm (overlap may occur) and is further identified by separations ($^2J_{(HH)}$, 12.5 - 14.5 Hz) of magnitudes intermediate between those of the other two systems (Fig. 3.1, p.41, shows a typical example). In such cases care is needed to distinguish the three AB doublet pairs especially as part of the peaks might overlap each other, or be obscured by the HOD peak when D_2O is used as solvent, or might partially overlap other signals, as in the case of moxalactam (Fig. 3.3 below) where the outer peak of the 3-CH_2S- signal overlaps that of the NMe. The position and coupling constant values of these doublets in D_2O, can have a role in the distinction of the various cephalosporins as shown in Table 3.8 (p.46).

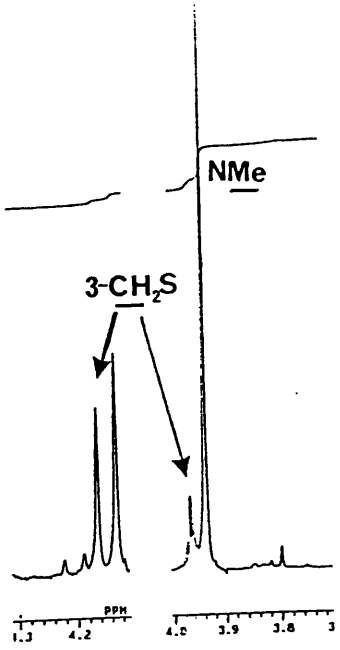


Fig. 3.3 : Partial 270 MHz ^1H NMR spectrum (expansion) of moxalactam (di-ammonium salt in D_2O) illustrating the overlap of part of 3-CH_2S signal with that of NMe. (Experimental conditions as in section 3.3.1)

Table 3.8 : δ (ppm) and J (Hz) values of the various 3-CH_2X signals of cephalosporins

X	Example	δ range	^2J value (Hz)
-OH	desacetylcephalothin	4.0 - 4.50 dd	~ 13.0
-OCOMe	cefotaxime	4.70 - 5.15 dd	12.5 - 13.5
$-OCONH_2$	cefoxitin	4.60 - 4.90 dd	12 - 13
-SAr	ceftriaxone	3.95 - 4.50 dd	13.5
$-C_5H_5N^+$	cefsulodin	5.30 - 5.65 dd	14.5

dd = double doublet, Ar = Aromatic.

From the Table above, the doublet pair signal due to 3-$CH_2N^+C_5H_5$ is lower field (5.30 - 5.65 ppm) compared to the resonances of the other 3-CH_2-X types, due to the greater deshielding effect of the positively charged nitrogen of the pyridyl ring; the doublets are further characterised by their relatively higher separations (^2J, 14.5 Hz). The AB doublet pair signal of the 3-CH_2X is observed at a much lower field than that of 2-CH_2 because of the greater deshielding effect of the adjacent X group, in addition to the effect of the olefinic group (the $\Delta^{3:4}$ double bond).

c) 3-X type derivatives

In these examples X is a group other than alkyl, e.g., chlorine as in cefaclor, or hydrogen as in ceftizoxime. Spectra are characterised by the absence of the 4-line AB signal, due to 3-CH_2X, in the region 4.0 - 5.5 ppm, and in most cases the absence of signals above 3.0 ppm. Furthermore, ceftizoxime shows a double doublet (dd) near 6.27 ppm (Fig. 3.4 below) with separations 6.3 and 2.0 Hz. These couplings are also apparent on the 2-CH_2 signals (dd), [Table 3.1].

Fig.3.4 : Partial ^1H NMR spectrum of ceftizoxime Na in D_2O showing the dd near 6.27 ppm due to C_3-H.

d) 3-CH=CH_2 type

This is represented by cefixime (Fig. 3.5 below and Table 3.1, p.53). The compound is characterised by the signals due to the -CH_a=CH_bH_c group, resonating as double doublet centred at 6.73 ppm (11.2, 17.5 Hz) for H_a, doublet at 5.42 ppm (17.5 Hz) for H_b and doublet centred at 5.24 ppm (11.2 Hz) for H_c.

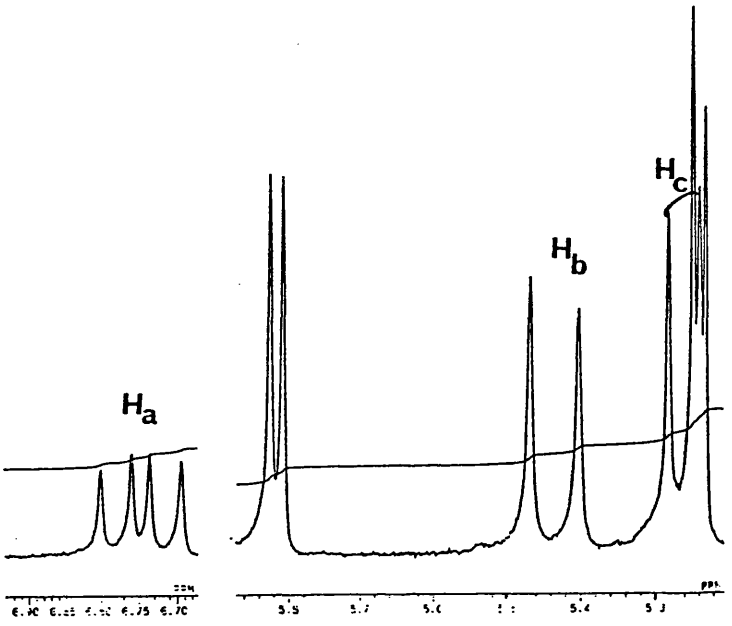


Fig. 3.5 : Partial ^{1}H NMR spectrum (at 270 MHz, in D_2O) of cefixime Na showing signals due to the -CH_a=CH_bH_c group. (Experimental conditions as in section 3.3.1).

e) ArCH, $ArCH_2$ type

The methine group attached to aromatic rings (ArCH-), such as in cephalexin, cephamandole and cefsulodin, or attached to a cyclohexadiene ring as in cephradine, shows a narrow one-proton singlet which is often situated in between the two β-lactam doublets (range 4.50 - 5.30 ppm). The position of the peak depends on the nature of the groups attached to the methine group, as was the case with cephamandole nafate [Table 3.1, p.54], where the methine peak appeared further downfield (6.20 ppm) due to the greater deshielding effect of the (OCHO) group substituted on the methine group. Furthermore, the solvent used might affect the peak position, especially in compounds having a β-amino group substituted

on the methine group, such as cephalexin; the observed chemical shift value (5.28 ppm) in D_2O-TFA (Table 3.1) is much lower than that reported in the literature (4.59 ppm). This is because in our study the β-amino group was protonated in the presence of TFA (NH_3^+ has a greater deshielding influence than NH_2); in the literature study , Na_2CO_3 was used to dissolve the sample thus the amino group was not ionised in alkaline solution.

The methylene protons of $ArCH_2$ type, may either give rise to a sharp two-proton singlet in the range 3.80 - 5.60 ppm, depending on the nature of the aromatic ring, e.g. cefoxitin (3.94 ppm) and cephazolin (5.55 ppm) [Table 3.1], or show an AB doublet pair resonance ranging from 3.80 - 3.90 ppm (J ~ 16.0 Hz), such as in cephalothin and cephaloridine. This doublet pair signal is differentiated from the other AB systems by the smaller chemical shift separations (0.05-0.10 ppm, Fig. 3.1, p.41).

2. Signals to lower field of 6 ppm

a) Aromatic signals

Low field signals due to the aromatic protons occur in all spectra of the cephalosporins listed in Table 3.1 (pp.51-54), except that of cephradine which displays a vinylic resonance (5.77 ppm, s, 2-proton, and 6.18 ppm, s, 1-proton). Ceftizoxime and cefixime also have vinylic resonances (plus the aromatic signals). The multiplicity of these aromatic signals can be used for identity purposes. Hence, by examining the nature of the aromatic proton signal which usually falls between 7 and 8 ppm the approach for identifying these cephalosporins can be summarised below:

(i) Phenyl type, as for cephalexin, cefaclor, cephamandole and cefsuldin. At lower magnetic fields (60 and 100 MHz) they give rise to a sharp five-proton singlet in the region 7.35 - 7.45 ppm, but at higher fields (270 and 400 MHz) they appeared as multiplets ranging from 7.35 to 7.60 ppm, due to resolution of short and long couplings between the five-protons [Table 3.1].

(ii) p-HOC_6H_4–R type, represented by cefadroxil, moxalactam and cefatrizine. They are identified by their characteristic AB doublet pair (2-proton intensity) resonances in the aromatic region of their spectra, centred at 6.85 - 6.90 ppm and 7.20 - 7.30 ppm (J=8.5 - 9.0 Hz) [Table 3.1], with additional lines due to long range coupling. This pattern is typical of p-disubstituted benzene ring [100]. The coupling constant is characteristic of coupling between aromatic protons situated ortho to each other.

(iii) Thienyl type, e.g cephalothin, cephaloridine and cefoxitin. These show a two-proton doublet at 7.0 - 7.05 ppm and a one-proton triplet at 7.28 - 7.37 ppm, for the thiophene ring, with additional lines due to long range coupling. This pattern is typical of 2-substituted thienyls.

(iv) Furyl aromatic type, as in cefuroxime. The pattern observed [Table 3.1, p.53] is characteristic of 2-monosubstituted furyl derivatives[100]. The signal at the lowest field is a one-proton apparent doublet centred at 7.68 ppm (J=1.8 Hz) which showed evidence of further long range splitting (as small inflexions on the doublet) as observed in the 400 MHz spectrum (Fig. 3.6, p.50). Upfield of this signal is a one-proton doublet centred at 6.88 ppm with a larger separation (J=3.7 Hz) and at the highest field is a doublet of doublets ranging from 6.61 - 6.63 ppm (J=3.70 and 1.80 Hz). The signals were attributed to $C_{5'}$-H, $C_{4'}$-H and $C_{3'}$-H, respectively. The larger coupling constant (J=3.7 Hz) is most probably due to the ortho coupling between the $C_{4'}$- and $C_{3'}$- protons, whilst the smaller coupling (J=1.80 Hz) may be attributed to meta coupling between the $C_{5'}$- and $C_{3'}$- protons[91].

(v) Aromatic singlets type, cefotaxime, ceftriaxone and cephazolin are identified by the simplicity of the 6-10 ppm region of the ^{1}H NMR spectra displaying a one-proton singlet at 6.97, 7.00 and 9.30 ppm, respectively [Table 3.1].

(vi) Signals due to pyridyl protons : These are of great diagnostic value as they appear lower than 8.00 ppm, as additional low field resonances. Spectra of cephaloridine and cefsulodin

are distinguished by their thienyl and phenyl proton resonances, respectively; furthermore, they are identified by their additional lower field resonances (8.0 - 10.0 ppm) due to the pyridyl protons (Fig. 3.7, p.50).

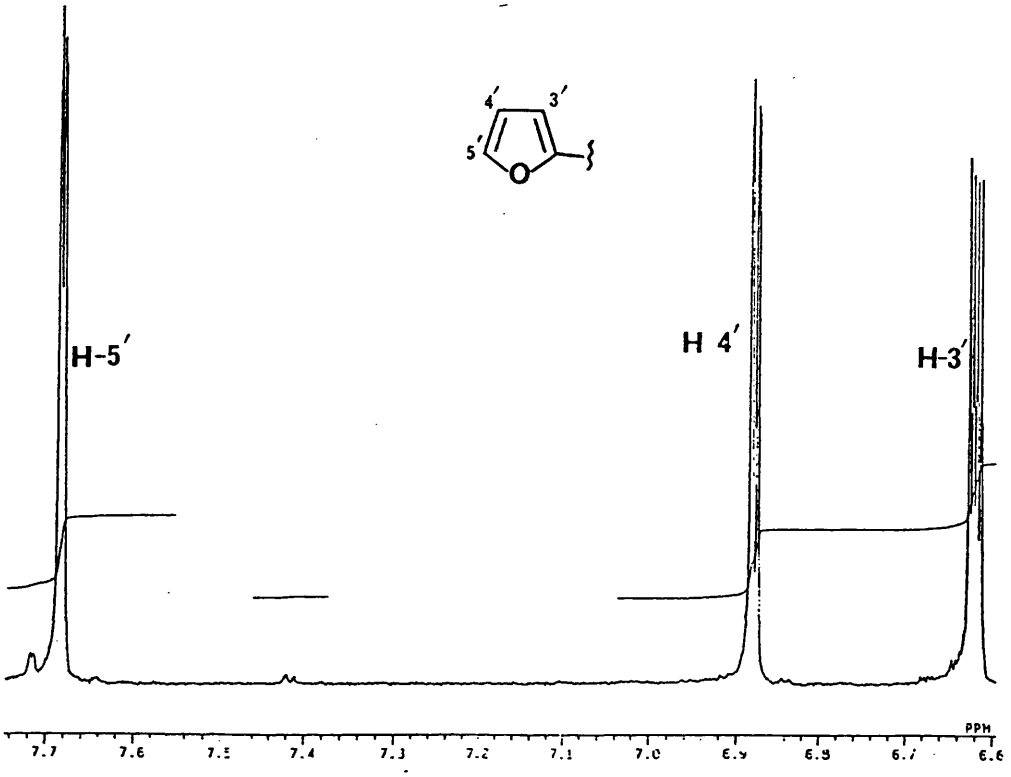


Fig. 3.6: Expansion of 400 MHz ^{1}H NMR spectrum of cefuroxime Na in D_2O (the region 6.60 - 7.75 ppm) to illustrate the signals due to furyl ring protons. See text (p.49) for details. (Experimental conditions as in section 3.3.1).

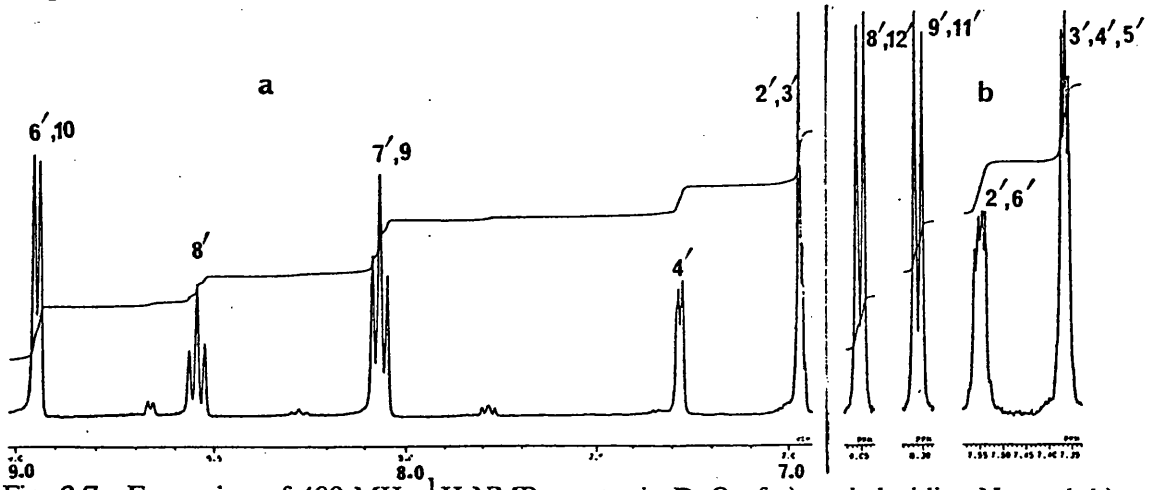
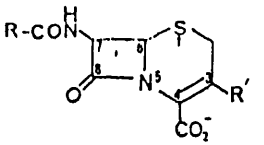
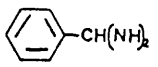
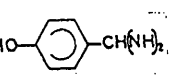
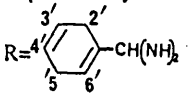
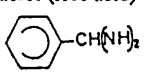


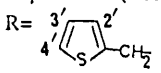
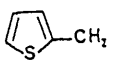
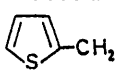
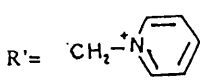
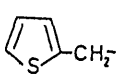
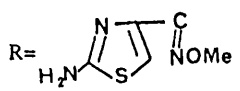
Fig. 3.7 : Expansion of 400 MHz ^{1}H NMR spectra in D_2O of a) cephaloridine Na and b) cefsulodin Na showing the aromatic region (7.0-9.0 ppm). See text p.49 for details. (Experimental conditions as in section 3.3.1).

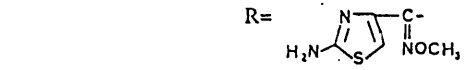
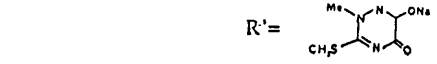
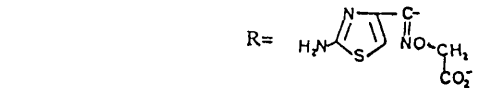
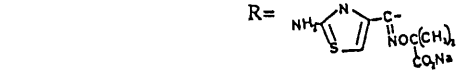
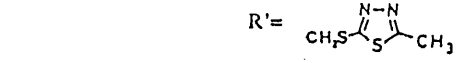
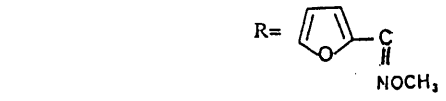
Table 3.1 :1H NMR characteristics of some cephalosprins (salts and free acids).

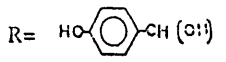
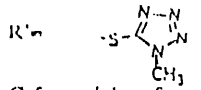
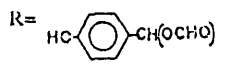
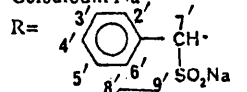
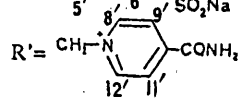
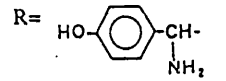
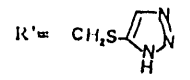
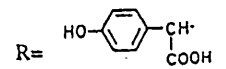
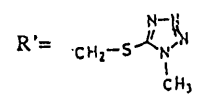


(continued overleaf)

Compound and form	Solvent[a] O.F. Ref.	2-CH_2	6-H, 7-H	Ar of R	Miscellaneous	Reference(s)
Cephalexin (free acid)	D_2O-TFA					
R= 	270	3.16, 3.42 d (18.7)	4.97, 5.60 d (4.4)	7.51-7.57 m	3-Me 2.07 s ArCH 5.28 s	**90, 91**
	400	3.15, 3.42 d (18.6)	4.96, 5.64 d (4.4)	7.51-7.55 m	3-Me 2.07 s ArCH 5.28 s	
R'= Me	DSS					
Cefadroxil (free acid)	D_2O-TFA					
R= 	270	3.04, 3.34 d (18.5)	4.86, 5.53 d (4.4)	6.87, 7.31 d (8.7)	3-Me 1.94 s ArCH 5.10 s	**90**
	400	3.02, 3.31 d (18.6)	4.84, 5.50 d (4.6)	6.84-6.86 m 7.27-7.29 m	3-Me 1.92 s ArCH 5.08 s	
R'= Me	HOD					
Cephradine hydrate	D_2O					
R= 	270	3.36, 3.60 d (18.7)	5.14, 5.68 d (4.4)		3-Me 2.15 s, RCH 4.68 s 3',4'-H 5.77 s, 6'-H 6.18 s 2',5'-protons 2.69-2.95 m	**90,91**
	400	3.35, 3.59 d (18.3)	5.13, 5.67 d (4.4)		3-Me 2.14 s, RCH 4.67 s 3',4'-H 5.76 s; 6'-H 6.17 s 2',5'-H 2.67-2.79 m	
R'= Me	DSS					
Cefaclor (free acid)	D_2O-TFA					
R= 	270	3.31, 3.72 d (17.7)	5.04, 5.64 d (4.8)	7.45 s (5H)	ArCH 4.88 s	
R'= Cl	HOD					

Cephalothin (free acid)	DMSO-d_6					
R= 	270	3.40, 3.60 d (18.0)	6-H 5.03 d (4.8) 7-H 5.70,5.73 dd (4.8, 8.1)	2',3'-H 6.92-6.95 m 4'-H 7.20-7.22 m	$ArCH_2$ 3.77,3.84 d(14.5) 3-CH_2 4.81,5.07 d (13.2) NH 9.06 d (8.1)	
R'= CH_2OCOCH_3	TMS					
Cephalothin Na	D_2O					
R= 	270	3.36, 3.63 d (17.9)	5.11, 5.64 d (4.8)	2',3'-H 7.04 d 4'-H 7.37 t	OCOMe 2.10 s $ArCH_2$ 3.91 d 3-CH_2O 4.70,4.87 d(12.5)	
R'= CH_2OCOCH_3	400	3.32, 3.58 d (18.0)	5.06, 5.60 d (4.9)	2',3'-H 7.00 m 4'-H 7.33 m	OCOMe 2.06 s $ArCH_2$ 3.83, 3.89 d(15.7) 3-CH_2O 4.67,4.83 d (12.5)	
	DSS					
Cephaloridine Na	D_2O					
R= 	400	3.12, 3.56 d (17.9)	5.11, 5.65 d (4.9)	2',3'-H 7.00 m 4'-H 7.32 m	$ArCH_2$ 3.81,3.86 d (15.9) 3-CH_2N 5.32,5.54 d (14.5) C_5H_5N+ 8.06,8.54 m 8.95 d (6)	**83, 84, 90 & 94**
R'= 	DSS					
Cefoxitin Na	D_2O					
R= 	270	3.22, 3.57 d (18.0)	6-H 5.12 s 7-OMe 3.51 s	2',3'-H 7.04 m 4'-H 7.36 m	$ArCH_2$ 3.94 s 3-CH_2O 4.63, 4.79 d(12.5)	**90, 93**
R'= CH_2OCONH_2	400	3.22, 3.57 d (18.0)	6-H 5.12 s 7-OMe 3.51 s	2',3'-H 7.03 m 4'-H 7.36 m	$ArCH_2$ 3.94 s 3-CH_2O 4.63, 4.79 d(12.7)	
7–OMe	DSS					
Cefotaxime Na	D_2O					
R= 	270	3.40, 3.67 d (18.0)	5.21, 5.82 d (4.8)	ArH 7.00 s	OCOMe 2.10 s, NOMe 4.00 s 3-CH_2O 4.72, 4.89 d (12.4)	
R'= CH_2OCOCH_3	400	3.40, 3.67 d (17.9)	5.21, 5.82 d (4.6)	ArH 7.00 s	OCOMe 2.10 s, NOMe 4.00 s 3-CH_2O 4.72, 4.89 d (12.7)	
	DSS					

Ceftizoxime Na	D_2O					
R= 	400	2-H_a 3.61, 3.66 dd (2.0, 18.4)	5.15, 5.81 d (4.7)	ArH 6.97 s	$NOCH_3$ 3.94 s	
R'= -H		2-H_b 3.41, 3.46 dd (6.3, 18.8)			C_3-H 6.26, 6.28 dd (2.0, 6.3)	
	HOD					
Ceftriaxone Na	D_2O					
R= 	400	3.44, 3.70 d (18.0)	5.16, 5.75 d (4.7)	ArH 6.97 s	NMe 3.58 s, $NOCH_3$ 3.94 s	
R'= 	HOD				3-CH_2S 4.02, 4.32 d(13.6)	
Cefixime +$NaHCO_3$	D_2O					
R= 	270	3.60, 3.73 d (17.4)	5.24, 5.81 d (4.7)	ArH 7.05 s	$NOCH_2$ - 4.57 s, H_c 5.26 d(11.2) H_b 5.43 d(17.5), H_a 6.75 dd(11.2,17.5)	
R'= -CH=CH_2	400	3.58, 3.70 d (17.3)	5.21, 5.79 d (4.6)	ArH 7.02 s	$NOCH_2$ 4.54 s, H_c 5.23 d(11.4) H_b 5.41 d (17.7), H_a 6.72 dd(11.4,17.7)	
	HOD					
Ceftazidime Na	D_2O					
R= 	400	3.25, 3.69 d (18.0)	5.33, 5.91 d (4.9)	ArH 6.98 s	$NOC(Me)_2$ 1.519s, 1.512s 3-CH_2 5.38, 5.63 dd(14.5) C_5H_5N+ 8.12m, 8.61m, 9.0m	
R'= 	HOD					
Cefazolin Na	D_2O					
R=	400	3.43, 3.79 d (17.7)	5.10, 5.69 d (4.7)	ArH 9.29 s	$ArCH_3$ 2.72 s, NCH_2 5.55 s 3-CH_2S 3.96, 4.50 d(13.7)	**90, 92**
R'= 	DSS					
Cefuroxime Na	D_2O					
R= 	270	3.40, 3.66 d (18.0)	5.21, 5.81 d (4.8)	2' 6.62m, 3' 6.88d 4' 7.68 d	NOMe 3.99 s 3-CH_2O 4.67,4.86 d(12.5)	**90**
	400	3.41, 3.66 d (18.0)	5.21, 5.81 d (4.7)	2' 6.62 dd(1.8,3.7) 3' 6.88 dd (0.6,3.7) 4' 7.68 dd (0.6,1.8)	NOMe 4.0 s 3-CH_2O 4.67,4.86 d(12.5)	
R'= -CH_2OCONH_2	DSS					

Cefamandole Li	D_2O					
R= 	270	3.33, 3.67 d (17.6)	5.02, 5.56 d (4.6)	5H- 7.38-7.48 m	NMe 3.98 s, ArCH- 5.24s 3-CH_2S 3.97,4.26 d(13.6)	**90, 93**
R'= 	400	3.34, 3.68 d (17.8)	5.03, 5.57 d (4.4)	5H 7.40-7.48 m	NMe 3.99s, ArCH 5.26s 3-CH_2S 3.98,4.27 d(13.7)	
	DSS					
Cefamandole nafate	D_2O^b					
R= 	400	3.30, 3.62 d	5.01, 5.8 d (4.6)	5H 7.37-7.53 m	NMe 3.97s, OCHO 8.28 d 3-CH_2S 3.98,4.24 d ArCH 5.26s (base), 6.17s (nafate)	**90**
R'=as for cef. Li	DSS					
Cefsulodin Na	D_2O					
R= 	400	3.02, 3.51 d (18.0)	5.11, 5.67 d (4.7)	3'to5'- 7.36-7.37m 2',6'- 7.55m	ArCH 5.15s, 3-CH2 5.30,5.55 d(14.5) Ar-9',11' 8.31 d(6.4) Ar-8',12' 9.05 d(6.4)	
R'= 	HOD					
Cefatrizine (in propylene glycol)	D_2O^b					
R= 	400	3.18, 3.61 d (17.9)	5.25, 5.83 d (4.9)	2',6'- 8.04t 3', 5'- 8.93d(5.8)	3-CH_2S 5.30, 5.55 dd (14.5) ArCH 6.9s, ArH 8.53t CH_3 (solvate) 1.44 d	
R'= 	HOD					
Moxalactam NH_4	D_2O					
R= 	270	4.37, 4.52 d (17.3)	6-H 5.11s 7-OMe 3.43	6.85d, 7.23d (8.5)	NMe 3.97s, ArCH 4.50s 3-CH_2S 4.01, 4.18 d (13.6)	
R'= 	400	4.36, 4.53 d (17.2)	6-H 5.07s,5.09s 7-OMe 3.40s, 3.49s	6.83m, 7.22m	NMe 3.95s, 3.97s ArCH 4.43s, 4.47s 3-CH_2S 3.95,4.16 d (13.4) 3.95,4.22 d(13.4)	
(1-O-analogue)	HOD					

a Solvent : D_2O= Deuterium oxide, TFA= Trifluoroaceticacid, DMSO-d_6= Deuterated dimethylsulfoxide. O.F.= Operating frequency in MHz; Ref.= Reference signals : HOD at 4.80 ppm, DSS at 0.00 ppm, and TMS at 0.00 ppm. b D_2O-Na_2CO_3

3.4.2 Identification scheme for cephalosporins based upon ^{1}H NMR data

It is clear from the discussion in the previous section and from the spectral data of Table 3.1 (pp.51-54) that each individual cephalosprin has its own specific ^{1}H NMR features. Hence, in this section, an analytical scheme, based upon ^{1}H NMR information, is devised for the identification of cephalosporin analogues having an intact β-lactam ring, in the single drug form rather than in a formulation.

Assuming we have one of the cephalosporins in Table 3.1 as an unknown then before recording the ^{1}H NMR spectrum, its solubility properties need to be established.

Step 1 : Solubility :

a) If the material is sufficiently soluble in deuterium oxide (D_2O) it is probably an alkali-metal salt.

b) If it is sparingly soluble in D_2O, but freely dissolves on acidification (add few drops of trifluoroacetic acid, TFA), a cephalosporin with an amino substituent is indicated.

c) If it is insoluble or sparingly soluble in D_2O and a dilute acid fails to promote dissolution, but a dilute alkali dissolves it, then it is probably a free acid.

d) If the substance is insoluble in D_2O, and a dilute acid or alkali fails to bring about dissolution, it must be an ester and will require $CDCl_3$ or DMSO-d_6 for solubility.

Having recorded the ^{1}H NMR spectrum of the sample in the appropriate solvent :

Step 2 : Presence of the common ^{1}H NMR spectral features of Δ^3-cephalosporin.

Key points in the identification of cephalosporins from the ^{1}H NMR spectra are the presence of the following characteristic features common to all Δ^3-cephalposporins :

(a) 6-H, 7-H AB doublet pair resonance in D_2O in the range 4.75-5.20 ppm and 5.50-6.00

ppm, respectively, with ^3J values of 4-6 Hz characteristic of cis protons in an intact lactam ring, and relatively large chemical shift separations (0.5-0.9 ppm). One exception, is the case of cefoxitin with a methoxy group replacing the proton at position-7; here the 6-H resonates as a singlet at 5.10-5.12 ppm, confirmed by the presence of a sharp three-proton singlet at 3.50 ppm (also in the case of moxalactam, see group 4 below).

(b) 2-CH_2 4-line AB signal in the region 3.0-3.9 ppm of separation 18 Hz, due to the non-equivalent methylene protons of the dihydrothiazine ring (which is lower field in moxalactam).

Having identified features (a) and (b) for the presence of a cephalosporin, specific evidence of identification among this group may now be sought.

Step 3 : For the purpose of simplifying the identification , the cephalosporins are divided into 4 groups, depending on similarity in chemical structure, and on the spectral features of substituents at C-3 and C-7 :

Group 1 : These are the amino derivative cephalosporins (detected by their solubility properties), e.g., cephalexin, cefadroxil, cephradine, cefaclor and cefatrizine. The first 3 compounds are characterised by having a sharp three proton singlet in the range 1.90 - 2.15 ppm assigned to the vinylic 3-methyl group; cefaclor may be distinguished by the absence of any signal upfield to 3 ppm; cefatrizine displays an additional AB doublet pair resonance (5.30, 5.60 ppm, J= 14.5 Hz) due to the CH_2S at position 3 . Cephalexin and cefaclor spectra are quickly identified by their 5-proton aromatic singlet (broad at base) near 7.50 ppm. Cefadroxil displays its typical AB aromatic resonance (two doublets centred at 6.85 and 7.30 ppm, J=9 Hz) as does cefatrizine. Cephradine's spectrum shows diagnostic broad signals at 5.80 and 6.20 ppm due to vinylic methylene of the cyclohexadiene grouping. The spectrum of cefatrizine may be distinguished by having additional peaks due to the solvate, propylene

glycol ($CH_3CHOHCH_2OH$), with an intense broad singlet at 1.40 - 1.45 ppm due to the Me group and the further splitting of the peaks due to the aromatic protons in the region 8.0 - 9.0 ppm.

Group 2: These are the 7-(2'-thienyl)acetamido-Δ^3-cephalosporin analogues, e.g., cephalothin, cephaloridine and cefoxitin; their spectra are well distinguished by a one-proton triplet at 7.34 ppm and a two-proton doublet at 7.00 ppm from the thienyl ring. The Ar-methylene group gives rise to a two-proton singlet at 3.95 ppm for cefoxitin, and an AB doublet pair resonance centred at 3.88 ppm (J=16 Hz) for both cephalothin and cephaloridine. The latter is identified by its additional low field resonance (8 - 10 ppm) due to the pyridyl protons, while the singlet 6-H (5.12 ppm) and C_7-OMe (3.51 ppm) resonances of the cefoxitin spectrum are likewise diagnostic.

Group 3: Spectra of cefotaxime, ceftriaxone, ceftizoxime, cefixime and cephazolin are characterised by the simplicity of the 6-10 ppm region, displaying only a sharp one-proton aromatic singlet at 7.00, 6.97, 6.97, 7.02 and 9.30 ppm, respectively. Both cefotaxime and ceftriaxone display a sharp three-proton singlet at 4.00 ppm for the -OCH_3 group ; the former displays another Me signal at 2.10 ppm and a 3-CH_2O AB doublet-pair resonance centred at 4.72 ppm, 4.90 ppm (J=12.5 Hz); The latter is distinguished by a CH_2S AB signal (4.02, 4.32 ppm; J=13.6 Hz) and a sharp 3-proton singlet at 3.60 ppm, due to the N-Me group. Ceftizoxime is distinguished by the double doublet near 6.2 ppm, with separations 6.3 and 2.0 Hz due to the vinylic C_3-H signals , cefixime by the vinylic resonance at 5.24, 5.42 and 6.73 ppm due to the 3-$CH{=}CH_2$ group.

Group 4: This group consists of the cephalosporins of Table 3.1 (pp.51-54) not mentioned in the previous groups. These are : cefuroxime, cephamandole, cefsulodin and the 1-O-analogue moxalactam. Their spectra are differentiated from those of the previous groups by aromatic

signal multiplicities to low field of 6 ppm. Cefuroxime displays a one-proton doublet at 7.68 ppm (J=1.8 Hz), one-proton doublet at 6.88 ppm (J=3.7 Hz) and a one-proton double doublet centred at 6.62 ppm (J=1.8 and 3.7 Hz). The distinguishing feature of cefsulodin spectrum is a low field complex : a two-proton doublet at 9.05 ppm (J=6.5 Hz), a two-proton doublet at 8.31 ppm (J=6.5 Hz) (due to pyridyl protons), a two-proton multiplet centred at 7.55 ppm and a three-proton multiplet centred at 7.36 ppm (due to phenyl protons). Cephamandole shows a broad five-proton multiplet in the range 7.38-7.48 ppm; moxalactam two 2-proton doublets centred at 6.85 and 7.23 ppm (J=8.5 Hz) (ArA_2B_2 system). Both these features are also displayed by some of group 1 cephalosporins (amino derivatives). The solubility properties and other spectral features allow the differentiation of the two groups. Of these, cephamandole is identified by its additional AB doublet pair resonance (3.97, 4.27 ppm, J=13.6 Hz) due to the 3-CH_2S group, while the 6-H singlet (5.10 ppm), the C_7-OMe singlet (3.43 ppm) and the relatively low field AB doublet pair (4.37 and 4.52 ppm, J=17.3 Hz) of the 2-CH_2 characteristic of the 1-oxo-analogues, resonances of the moxalactam spectrum are likewise diagnostic.

The above analytical scheme of identification may only yield initial evidence of structure and must be confirmed by a complete spectral analysis ensuring that all the other resonance features accord, both in position and intensity, with the proposed structure.

3.4.3 ^{1}H NMR diagnostic features of cephalosporonate esters

The ^{1}H NMR spectra of Δ^3-cephalosporonate esters in deuterochloroform ($CDCl_3$) and deuterated dimethylsulfoxide (DMSO-d_6) [Table 3.2, p.63] exhibit the same common spectral features characteristic of cephalosporin analogues, i.e., the β-lactam ring protons and the thiazinyl ring 2-CH_2 protons resonances. In cephalosporonate esters, the 6-H and 7-H signals appeared as a single-proton doublet centred at 4.75 - 5.10 ppm and a single-proton double doublet in the range 5.60 - 6.00 ppm, respectively. The 7-H dd signal is due to coupling to 6-H (J=4-5 Hz) on one side and with the imino-proton (NH) on the other

(J=8.2-9.3 Hz). Depending on the nature of the solvent, the NH doublet position varies, appearing at 6.10 - 6.40 ppm (J=9.0 Hz) in $CDCl_3$ or at 9.12 ppm (J=8.24 Hz) in DMSO-d_6 [Table 3.2]. The 2-CH_2 signal shows the typical AB doublet pair in the range 3.10 - 3.70 ppm (J=18-18.5 Hz) with only one exception, the 7-ADCA p-nitrobenzyl ester in D_2O-TFA where it appeared as a broad singlet at 3.34 ppm [Table 3.2]. From this Table the main ^1H NMR spectral features for the identification of the cephalosporonate esters are summarised as follows:

1. p-Nitrobenzyl esters, represented by the 7-amino and 7-(2-phenyl acetamido) 3-methyl-4-p nitrobenzyl carboxylate Δ^3-cephem, and the esters of cephalothin and desacetylcephalothin. The spectra of these esters are characterised by the following diagnostic features :

a) Low field A_2B_2 doublet pair resonances, due to the aromatic protons of the p-nitrobenzyl group. The AB signal is clearly seen in the 7-ADCA ester spectrum in the range 7.26 - 8.25 ppm (J=8.6-8.9 Hz); the position and coupling separation values depending on the solvent used [Table 3.2, p.63]. In spectra of the other esters the signal appeared close to, but resolved from, the other aromatic signals. The phenyl protons of the phenylacetamido derivatives show a five-proton multiplet in the range 7.20 - 7.40 ppm, while those of the p-nitrobenzyl group appeared further downfield as well resolved two 2-proton doublets centred at 7.63 and 8.22 ppm (J=8.8 Hz) and separation 0.63 ppm (Fig. 3.8, p.61).

The 2'-thienylacetamido-esters give rise to two 2-proton doublets at 7.58 and 8.23 ppm (J=8.8 Hz) for the p-nitrobenzyl group aromatic protons, and upfield a one-proton triplet and two-proton doublet at 7.00 and 7.28 ppm, respectively, for the thiophene ring. Generally, the aromatic protons resonance of the p-nitrobenzyl group are lower field than the other aromatic signals, due to the greater deshieding influence of the nitro group.

b) Methylene signal of the p-nitrobenzyl group (OCH_2Ar) : The ester of 7-ADCA shows a sharp two-proton singlet at 5.37 ppm (in DMSO-d_6), a singlet overlapping the 7-H doublet at 5.07 ppm (inD_2O-TFA) and an AB doublet pair at 5.38 and 5.31 ppm (J=13.5 Hz) and a separation of 0.07 ppm(in $CDCl_3$). The phenylacetamido derivative displays a two-proton doublet pair resonance at 5.26, 5.36 ppm (J=13 Hz) in $CDCl_3$ and a sharp two-proton singlet at 5.40 ppm (in DMSO-d_6) for the methylene group. The cephalothin and desacetylcephalothin esters spectra (in $CDCl_3$) exhibit the AB doublets resonance for the methylene protons of the p-nitrobenzyl group centred at 5.34 ppm 5.39 ppm (J=13.0 Hz), respectively. The former is distinguished by having very narrow chemical shift separation compared to that of desacetyl cephalothin (0.10 ppm).

2. Diphenylmethyl esters, represented by 7-phenoxyacetamido-3- methyl-4-diphenylmethyl carboxylate Δ^3-cephem. The main diagnostic features of its spectra (Fig. 3.9, p.61) are:

a) A complex aromatic signal in the range 6.90 - 7.50 ppm having a large integral (16 protons); 15 from the three phenyl rings and ^1H of the methine group (-$CHPh_2$).

b) The relatively low field methine (CH) signal of the diphenyl methyl group, due to the combined deshielding effect of the two phenyl rings and the oxygen, obscured by the aromatic complex signal.

c) The methylene protons for the phenoxyacetamido-group of the ester give rise to a sharp two-proton singlet at 4.56 ppm (in $CDCl_3$) and two-proton double doublet at 4.68 and 4.61 ppm (J=13.5 Hz) in DMSO-d_6, Table 3.2.

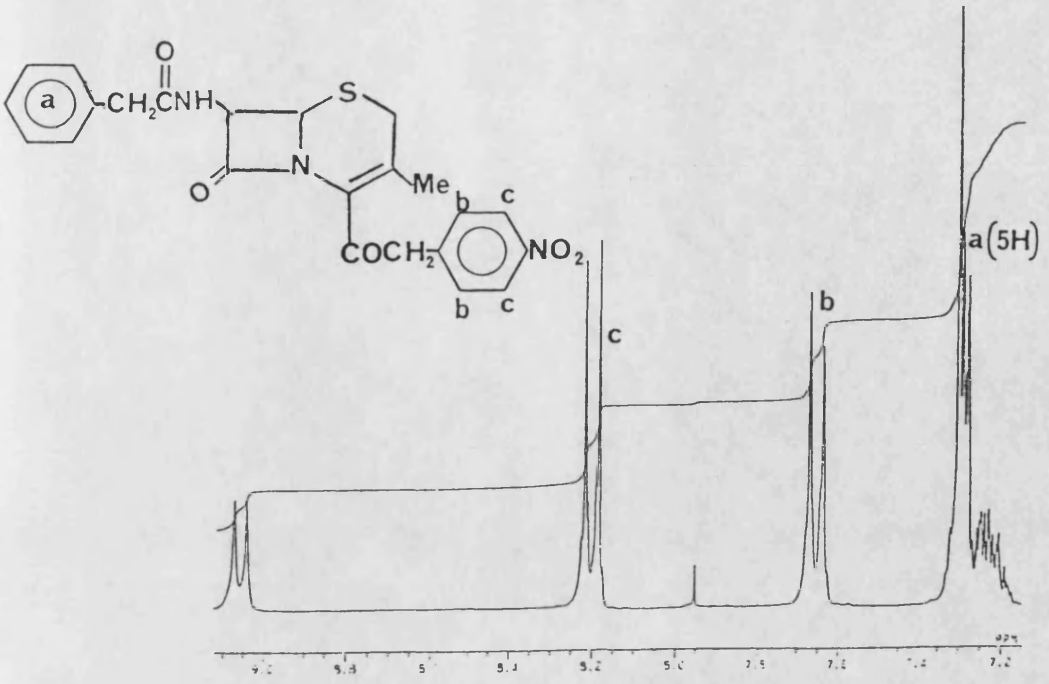


Fig 3.8 : Expansion of 270 MHz ^{1}H NMR spectrum of 7-phenylacetamido-3-methyl-4-p-nitrobenzylcarboxylate-Δ^{3}-cephem ($CDCl_3$) showing the aromatic region signals (7.0 - 9.0 ppm).

(Experimental conditions as in section 3.3.1)

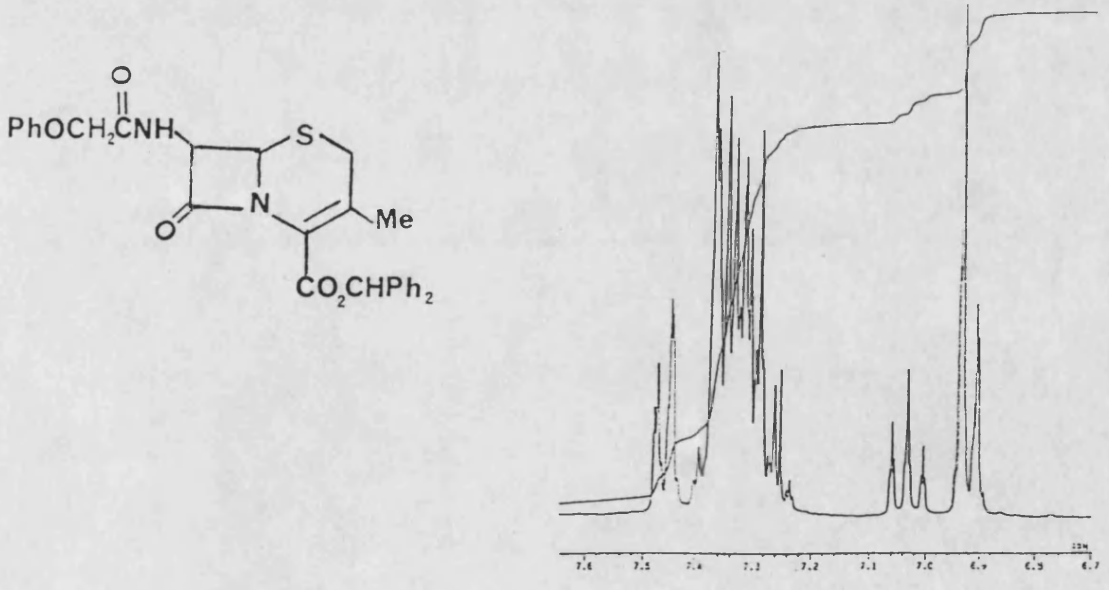


Fig 3.9 : Expansion of 270 MHz ^{1}H NMR spectrum of 7-phenoxyacetamido-3-methyl-4-diphenylmethylcarboxylate-Δ^{3}-cephem in $CDCl_3$ to illustrate the aromatic region (6.9 - 7.5 ppm). See text for details.

3.4.4 ^{1}H NMR characterisation of various cephalosporin derivatives

Table 3.9 below summarises the common ^{1}H NMR spectral features (i.e. 2-CH_2, 6-H and 7-H) of the cephalosporin salts, free acids, esters and lactones.

	Salts (D_2O)	free acids (D_2O-TFA)	esters ($CDCl_3$/ DMSO-d_6)	lactones (DMSO-d_6/ D_2O-TFA)
2-CH_2	3.00-3.90 d (17-18 Hz)	3.0-3.90 d (17.5- 19.0)	3.10-3.70 d (18.0-18.5)	3.45-3.95 d (18.0-19.0)
6-H	4.85-5.35 d (4.0-5.0)	4.84-5.15 d (4.0-5.0)	4.95-5.10 d (4.0-5.0)	5.20-5.30 d (4.0-5.0)
7-H	5.50-5.95 d (4.0-5.0)	5.0-5.75 d (4.0-5.0)	4.65-5.85, 5.7-5.9 dd(4-5, 8-9)	5.90-6.0 dd (4-5, 8.0-8.5)

From the above Table slight differences were observed between the various cephalosporin derivatives. Furthermore, the esters and lactones can be differentiated by their specific ^{1}H NMR spectral features [Tables 3.2, 3.3, respectively] as follows:

For esters

(1) The p-nitrobenzyl esters are characterised by the signal due to the methylene protons of the ester group. Depending on the solvent, it may appear as a two-proton singlet at 5.40ppm (in DMSO-d_6), or as double doublet (in $CDCl_3$) centred at 5.30 - 5.34 ppm with separation 13.0 Hz. In addition, the phenyl protons resonate as pair of doublets centred at 7.57 - 7.64 ppm and 8.20 - 8.24 ppm (separation 8.8 Hz).

(2) The diphenylmethyl esters show very complicated aromatic resonance in the range 6.90 - 7.60 ppm integrating for ten protons, in addition to the exceptionally lowfield singlet (>6.90 ppm) due to the methyl proton which is mostly obscured by the aromatic multiplet.

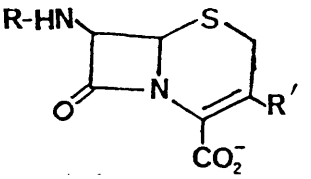
For lactones

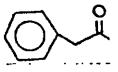
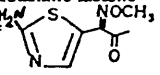
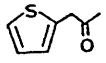
They are characterised by the two-proton singlet in the range 5.10 - 5.20 ppm due to the equivalent methylene protons of the lactone ring (see Table 3.3 p.64, and Chapter 4 section 4.4.4 for full account).

Table 3.2 : ^{1}H NMR chemical shifts of cephalosporin esters

Copmound and manufacturer	Solvent Reference (ppm) Operating freq.(MHz)	2-CH_2	6-H, 7-H	Aromatic protons	Miscellaneous
7-phenylacetamidodes-acetoxyceph.4-p-nitro-benzyl ester(Glaxo)	DMSO-d_6 TMS 270 0.00	3.45, 3.63 d (18.4)	6-H 5.09 d (4.8) 7-H 5.66, 5.69 dd (4.6,8.2)	PhCH2 7.25-7.31 m Ar-2',6' 7.69 d (8.6) Ar-3',5' 8.24 d (8.8)	3-Me 2.06s, $ArCH_2CO$ 3.54d(8.4) $ArCH_2$(ester) 5.40s CONH 9.12 d (8.2)
(formula p. 88)	$CDCL_3$ TMS 270 0.00	3.18, 3.50 d (18.4)	6-H 4.94 d (4.8) 7-H 5.78, 5.81 dd (4.8, 9.1)	PhCH2 7.26-7.39 m Ar-2',6' 7.57 d (8.8) Ar-3',5' 8.22 d (8.8)	3-Me 2.13s, $ArCH_2CO$ dd 3.65 ArCH2(ester) 5.26,5.36d(13.2) NH 6.12 d (9.2)
Cephalothin-4-p-nitro-benzyl ester (Bath) (formula p. 90)	$CDCL_3$ TMS 270 0.00	3.38, 3.57 d (18.5)	6-H 4.98 d (4.9) 7-H 5.85, 5.89 dd (4.9, 9.1)	Ar-2',3' 7.0 m (thienyl) Ar-4' 7.28 m (thienyl) Ar-2H 7.57 d (8.8)(ester) Ar-2H 8.23 d (8.8)(ester)	3'-Me 2.07 s,$ArCH_2$ 3.87 s 3-CH_2 4.80, 5.14 d (13.6) $ArCH_2$(ester) dd 5.34 NH 6.32 d (9.1)
Desacetylcephalothin-4-p-nitrobenzyl ester (Univ of Chicago) (formula p. 30)	$CDCL_3$ TMS 270 0.00	3.45, 3.66 d (18.0)	6-H 4.95 d (4.9) 7-H 5.86, 5.89 dd (4.9, 9.2)	Thienyl 7.0 m, 7.28 m Ester 7.58 d (8.8) 8.23 d (8.8)	$ArCH_2$ 3.87 s, NH 6.39 d(9.0) 3-CH_2 4.03, 4.48 d (13.0) $ArCH_2$(ester) 5.29, 5.39d (13.0)
4-p-Nitrobenzyl ester of 7-ADCA (Lilly)	DMSO-d_6 TMS 270 0.00	3.38, 3.62 dd (18.3)	4.77, 4.99 d (4.9)	Ar-2',6' 7.69 d (8.9) Ar-3',5' 8.20 d (8.9)	3-Me 2.02 s, $ArCH_2$ 5.37 s
7-Phenoxyacetamido-3-methyl-4-p-diphenyl-methylcephalosporin ester (Glaxo)	$CDCL_3$ TMS 270 0.00	3.19, 3.45 d (18.7)	6-H 5.00 d (4.8) 7-H 5.85, 5.88 dd (4.8, 9.2)	Ar-(15H) 6.90-7.48 m	3-Me 2.12 s, $PhOCH_2$ 4.56 s Ph_2CH obscured
(formula p. 90)	DMSO-d_6 TMS 270 0.00	dd at 3.53	6-H 5.15 d (4.6) 7-H dd 5.72	Ar-(15H) 6.91-6.99 m 7.27-7.54 m	3-Me 2.03 s, $PhOCH_2$ dd 4.64 NH 9.12 d (8.4), Ph_2CH obscured

Table 3.3 : ^{1}H NMR features of cephalosporin related compounds



Compound and form[a]	Solvent[b] O.F. MHz	2-CH_2	6-H, 7-H	Ar of R	Miscellaneous
7-ADCA (free acid)	D_2O-TFA	3.3, 3.4 d	4.89, 5.06 d		3-Me 2.02 s
R= H	270	(18.0)	(4.7)		
	Na salt in	3.20, 3.57 d	4.67 d, 5.0 t		3-Me 1.85 s
R′= Me	D_2O-$NaHCO_3$	(18.0)	and 5.35 d[c]		
	270				
2.8(free acid)	$CDCl_3$	3.10, 3.43 d	6-H 4.89d (4.8)	7.19-7.29 m	3-Me 2.09 s
R= 	270	(18.7)	7-H 5.68,5.72 dd (4.6,8.8)		Ar-CH_2 3.58 dd 7-NH 6.15d (8.8)
	Na salt in D_2O	3.18, 3.55 d	5.04, 5.55 d	7.36-7.40 m	3-Me 1.91 s
R′= $-CH_3$	270	(18.5)	(4.5)		$ArCH_2$ 3.64,3.72 d (14.6)
Cefotaxime lactone	D_2O-TFA	3.74, 3.93	5.31, 5.94 d	7.14 s	NOMe 4.08 s
R= 	270	(18.6)	(4.8)		lactone CH_2 5.11s
R′= 					
Cephalothin lactone	DMSO-d_6	3.49 s	6-H 5.23d (5.1)	2',3' 7.06-7.09 m	$ArCH_2$ 3.87,3.93 d (13.9)
R= 	270		7-H 5.95,5.98 dd (5.1,8.4)	4' 7.48-7.50 m	Lactone CH_2 5.17 s 7-NH 9.33d (8.4)
R′= 					

a 7-ADCA=7-aminodesacetoxycephalosporanicacid, -Δ^3-cephalosporin.

b (for abbreviations see footnote under Table 3.1).

c see text p. 65 for details.

2.8 =7-(2-phenylacetamido)desacetoxy

3.4.5 7-Amino desacetoxycephalosporanic acid (7-ADCA)

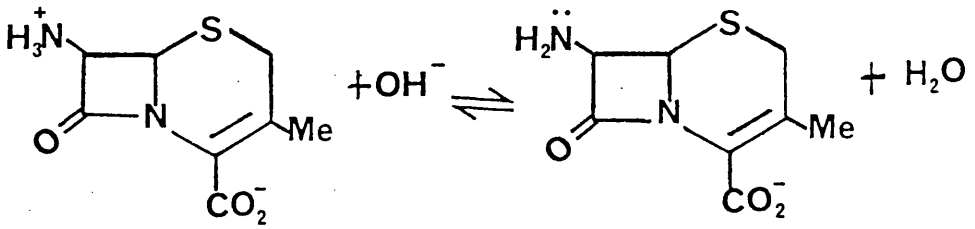
This compound is the precursor of many 3-methyl cephalosporins. It has a ^1H NMR spectrum [Table 3.3, p.64] that lacks signals below 5.1 ppm (as free acid in D_2O-TFA), and may be identified from this unique feature. The usual 6-H, 7-H and 2-CH_2 AB doublet pairs are present. Spectrum showed the 6-/ 7-H doublets centred at 4.9 and 5.06 ppm (J=4.7 Hz), and those of 2-CH_2 at 3.3 and 3.4 ppm (J=18.0 Hz), together with a three-proton singlet at 2.02 ppm, due to the 3-Me group. On storage of the solution at room temperature for 10 days, no change in the above spectral features was observed.

The sodium salt of 7-ADCA prepared from a mixture of the free acid and a small excess of $NaHCO_3$ in D_2O behaved anomalously. The doublet centred at 5.35 ppm, which is associated with 7-H, had only half the expected intensity while a second doublet with intensity corresponding to about half a proton was centred at 4.67 ppm. An apparent triplet was also present near 5 ppm made up of overlapping doublets. A single 4-line 2-CH_2 signal was present of greater AB chemical shift difference than found for 7-ADCA in D_2O/TFA, and a single Me resonance (1.88 ppm). Spectral appearance changed little on storage. These results may be interpreted in terms of the solution in D_2O-$NaHCO_3$ being : a) a mixture of the intact and β-lactam-opened product or a) a mixture of the two epimers of ring opened material in equilibrium. The absence of duplicate 2-CH_2 and 3-Me signals argues against either explanation. When the solution of 7-ADCA in D_2O-$NaHCO_3$ was acidified with DCl its spectrum reverted to that of a single product with features : 7-H, 6-H narrow doublets flanking HDO signal at 4.8 ppm, 2-CH_2 closely placed doublets centred near 3.15 ppm with inner lines of greater intensity than outer lines, 3-Me singlet ~ 1.8 ppm. This spectrum resembled that of 7-ADCA in D_2O-TFA prepared freshly, and also that of 7-ADCA in DMSO-d_6 plus DCl [Table 3.6]:

Table 3.6 : ^{1}H NMR characteristics of 7-ADCA in DMSO-d_6 and after addition of DCl

	7-H, 6-H	2-CH_2	3-Me
7-ADCA in DMSO-d_6	4.91d (4.9 Hz) 4.70d (4.9)	3.56d (19.2) 3.29d (18.3)	1.97s
plus DCl	5.17d (4.9) 5.05d (4.9Hz)	3.60d (17.4) 3.50d (17.4Hz)	1.93 s

These results are evidence that the spectral appearance of 7-ADCA in D_2O-$NaHCO_3$ is a result of the amino acid existing as a mixture of mono and di-ionized species at this pH which interconvert slowly on the NMR time scale.



di-ionized
(zwitterion)

mono-ionized
(anion)

The same NMR phenomenon was observed in the case of 7-aminocephalosporanicacid (7-ACA) as solution in D_2O-$NaHCO_3$: doublet near 5.4 ppm, two overlapping doublets near 5.0 ppm, doublet near 4.7 ppm.

3.4.6 ^{13}C NMR features of cephalosporin salts, free acids and esters

^{13}C NMR is another NMR technique valuable for the characterisation of cephalosporin derivatives[92,94,101–103]. Its advantages over ^{1}H NMR studies is the greater chemical shift range of ^{13}C over ^{1}H (^{1}H : 0 - 10 ppm, ^{13}C : 0 - 200 ppm) with the result that chances of resolving signals due to carbons in non-identical environments are high.

During the course of study described in this section, many ^{13}C NMR spectra of cephalosporin derivatives were recorded but comment is restricted to a few examples.

All the ^{13}C NMR spectra were run under proton decoupled conditions (see section 3.3.1, p.38). The assignments of the ^{13}C signals are based chiefly upon the expected chemical shift behaviour and on correlation with published data. One-bond and long-range coupling values to protons (obtained from coupled spectra) provide valuable evidence for carbonyl and other assignments, as will be discussed.

The following is an analytical scheme for interpreting the ^{13}C NMR spectra of the cephalosporin derivatives :

3.4.6.1 Common ^{13}C spectral features

1. The cephem ring carbons

The assignment of resonances for carbons directly bonded to hydrogen atoms (sp^3 type) were based on the DEPT technique (see section 3.3.1). The quaternary carbon frequences were identified by their absence in the 135° and 90° DEPT spectra :

(a) sp^3 type : C-7 and C-6 (CH positive from DEPT); C-2 (CH_2 inverted from DEPT).

From previous studies on free acids and alkali metal salts in D_2O and esters in $CDCl_3$ and DMSO-d_6[84,94,101–103], using the $^{1}J_{(C,H)}$ coupling magnitudes, and from the present study, the chemical shift rank orders are compared below :

Acids,salts and esters	range from previous studies (in ppm)	range from present results (ppm)*
C-7	57.1 - 64.2	57.0 - 61.4
C-6	55.5 - 63.4	54.4 - 58.0
C-2	24.3 - 31.6	23.0 - 30.3

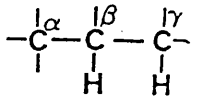
(* All compounds of Tables 3.4, 3.5, and those in section 3.4.8, p.88 except for moxalactam).

The chemical shift values of the present study agree well with those reported. Values outside these ranges, which are seen in case of moxalactam (C-7 94.0 ppm, C-6 82.2 ppm, C-2 64.8 ppm) are due to influence of the oxygen substituent. The $^{1}J_{(C,H)}$ values of C-6 and C-7 aid specific assignments. As C-6 is flanked with the electronegative neighbours sulphur (in position-1) and nitrogen (position-4), it is expected to possess a larger ^{1}J value than those of C-7 and C-2, as reviewed by Hansen[104]. This is illustrated by the coupled spectra of cefixime ($^{1}J_{(CH)}$ C-6 196 Hz, C-7 173 Hz) and ceftizoxime ($^{1}J_{(CH)}$ C-6 near 176 Hz, C-7 156 Hz) (Figs. 3.12, p.79, and 3.14, p.82, and Tables 3.10, p.80, and 3.11,p.81, respectively). Many cephalosporin spectra display additional signals near 60 ppm, which can often be distinguished from the ring carbon resonance by their features in coupled spectra,e.g. $NOCH_3$ of ceftizoxime 62.4 ppm (q, J=145.4 Hz) and $ArCHNH_2$ of cephalexin 59.3 ppm (d, J=157 Hz, no long range coupling).

(b) Sp^2 type : These are the quaternary carbons attached to double bonds (C-3 and C-4, C-8 carbonyl) which give signals that are absent in the 135° and 90° DEPT spectra.

The discrimination of the C-3 and C-4 signals (in ^{13}C NMR) is a controversial problem[103,105,106]. Dipole-dipole relaxation time (T_1) measurements have been used to aid

assignments of C-3 and C-4 : if it is assumed that the molecule is reorienting in an isotropic fashion and that the relaxation mechanism is essentially dipolar, the relaxation time of a quaternary carbon is expected to decrease with an increasing number of β-hydrogens[109] (Fig. 3.10 below) :



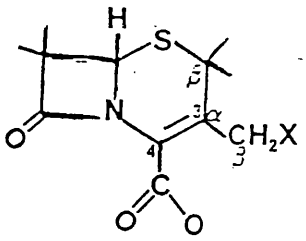


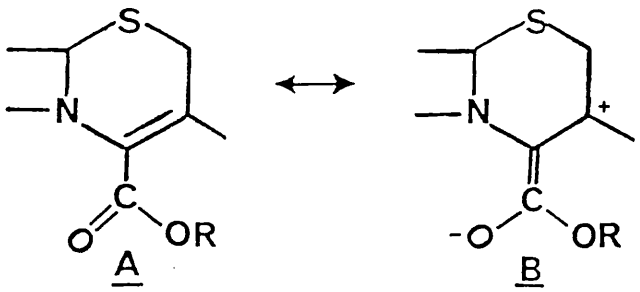
Fig.3.10 : Designation of protons in regard to carbon atom under consideration

Thus, C-3 in Fig. 3.10 has four β-protons and C-4 none; therefore, C-3 may be assigned upfield to the resonance of the less efficiently relaxed C-4, with the longer T_1 value. Neuss et al.[105,106] also concluded that C-4 resonates at lower field than C-3 but only on the basis of chemical shifts of model compounds. It was also observed that, in most cases, in ^{13}C NMR spectra of cephalosporin salts, the C-3 resonance is more intense than the lower field resonance of C-4[108,109], which is a very useful fact for distinguishing between these two signals. Some argue that C-3 is more shielded than C-4, so is the higher field signal[103]. Tori et al.[101] reported that for esters comparison of the C-3 and C-4 signal intensities (in $CDCl_3$), showed that the signal at the lower field was more intense than that of the higher field. When T_1 values were measured, the lower-field signal had the shorter T_1 value, and hence was assigned to C-3.

From present results, in spectra of cephalosporins with ionised 4-carboxylate functions [Table 3.4, p.74], ranges for C-3 and C-4 signals are 117.3-123.3 ppm (111.8 ppm for cefsulodin) and 127.0-131.4 ppm, respectively. The relative carbon assignments to both of these regions is confirmed by their proton-coupled spectra. Furthermore, the higher field

range is assigned to C-3 and the lower to C-4 (in case of salts) on the basis of a few T_1 measurements (e.g., ceftriaxone Na: 1.41 s for the 118.9 ppm signal, 3.48 s for the 130.2 ppm signal).

Our investigations on esters (section 3.4.8, p. 88) show that C-3 becomes the lower field resonance, a conclusion supported by the multiplicities of C-3 and C-4 in proton-coupled spectra (e.g. 4-p-nitrobenzyl ester of cephalothin, Fig.3.18, p. 91: C-4 125.0 ppm, broad singlet, C-3 127.0 ppm, multiplet) and signal intensities. The fact that the C-3 resonance is more deshielded than C-4 is possibly due to contributions from the resonance form B, of structure below which is unfavoured in the salt :



2. The carbonyl carbons

These are the 4-CO_2X, 7-amido, and lactam ring (C-8) carbonyl carbons which are common to all cephalosprin derivatives.

Discrimination of the carbonyl carbon of the lactam ring (C-8) from the other carbonyl atom signals in cephalosporin spectra was also a problem. This is due in part to the difficulty of detecting the peaks at natural carbon-13 abundance, owing to their very low intensity and also to their proximity to each other in the carbonyl region of spectrum, i.e., 160-177 ppm. Other techniques were employed by some workers to assign these signals, such as single frequency selective heteronuclear decoupling (s.f.s.d.)[110], off-resonance proton decoupling[103] and T_1 measurements[101]. Hence, C-8 was differentiated by considering the

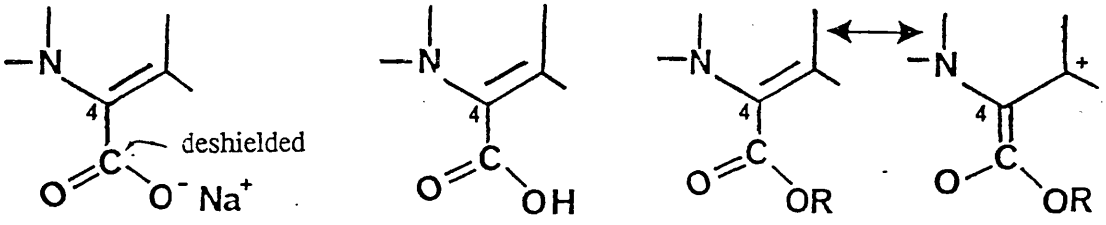
fine structure in the proton—coupled spectra, and from the signal intensities (and their T_1 values). C-8 has one β and one γ proton, appearing as a triplet with long-range coupling (^3J ~ 6 Hz)[105], C-10 (of CO_2X) has none, and splitting of the CONH line is mainly determined by the coupling with adjacent protons; furthermore, the CONH carbon was reported[103] to have the shortest T_1 and be the most deshielded, C-8 the less deshielded while CO_2X resonates between the two[101,103].

Results from the study of the various cephalosporin derivatives, i.e., salts, acids, and esters (Tables 3.4, 3.5, and sections 3.4.7, 3.4.8 respectively) confirm that the lactam (C-8), 4-carboxylate and 7-amido carbonyl resonances fall in a chemical range of 160.0-170.0 ppm. The data in the following Table summarises the range for each, compared to those appeared in literature :

	Literature data (ppm)[101]			present results (ppm)		
	salts	acids	esters	salts	acids	esters
7-amido	168-175	165-170	169-174	164-176	169-170	168-171
4-CO_2R	164-171	163-171	162-163	164-169	164-166	161-162
8-lactam CO	163-170	161-168	164-169	161-164	161-166	164-165

The carbonyl carbon resonances are easily identified from coupled spectra, supported by a few T_1 measurements. In all coupled spectra the lactam carbonyl signal (C-8) resonates as a narrow triplet due to coupling to C-7 and C-6 protons. The 4-carboxylate carbonyl resonates as a broad singlet near 169 ppm for alkali metal salts in D_2O (ionised, deshielded), 165 ppm for free acids (non-ionised carboxylate function), and 162 ppm for esters (due to resonance form B shown above). These variations in chemical shifts of the 4-CO_2X carbon

resonance reflects the greater deshielding influence of COO^- over that of CO_2H or CO_2R[113], the 4-C=O chemical shift of the salt being lower field than that of free acids and esters (as is illustrated by the structures below) :



salt free acid ester

It is observed, in most cases, that the 7-amido C=O resonates as the lowest field signal (in the range 170 - 175 ppm) and forms a multiplet in coupled spectra.

Generally, in salts and acids, the 7-CONH resonates as the lowest field signal, the lactam C=O the highest while 4-CO_2X resonates between the two. In cases of esters : amido the lowest, 4-CO_2R the highest and lactam resonating between the two.

3.4.6.2 Specific ^{13}C spectral features

1. Signals to higher field of 100 ppm

a) 3-Me type (identified by DEPT), e.g. cephalexin, shows a signal near 20 ppm appearing as a quartet in the coupled spectrum [$^1J_{(CH)}$~ 130 Hz].

b) 3-CH_2X, another sp^3 carbon (CH_2, DEPT), which is differentiated from 2-CH_2 signal by its lower field position (e.g., cefsulodin displays a 3-CH_2 resonance near 62.0 ppm).

c) Me signal, in 3-CH_2OCOMe, appearing near 21.0 ppm.

d) 7-OMe type, resonating near 53.0 ppm (moxalactam).

e) NOMe type, compared to other Me signals, resonates as the lowest field (e.g., ceftizoxime shows an NOMe resonance at 62.4 ppm, ceftriaxone 62.5 ppm).

Signals due to features of substituents at C-3 and C-7 complicate the spectrum.

2. Signals to lower field of 100 ppm

a) Aromatic signals : Low field signals due to the aromatic carbons occur in all spectra of the cephalosporins listed in Tables 3.4 and 3.5 (except 7-ADCA), and those in sections 3.4.7 and 3.4.8, which provide diagnostic ^{13}C signals. According to the nature of the aromatic carbon signals the type of ring can be identified as briefly summarised below :

i) The phenyl or thienyl type (as in cephalothin, cefsulodin and cephalexin), the sp^2 carbons give rise to signals in the region 125.0 - 130.0 ppm and quaternary carbons 131.0 - 140.0 ppm (except for the phenoxy type which display a Cq signal at 155.0 - 158.0 ppm).

ii) Ceftizoxime, cefixime and ceftriaxone [Table 3.4] have only one CH sp^2 carbon in their thiazole ring which resonates near 113.0 ppm and the quaternary carbons in the range 140.0 - 148.0 ppm.

iii) Esters : the p-nitrobenzyl type shows resonances between 127.0 - 129.5 ppm for the CH sp^2 carbons and 141.0 - 143.0 ppm and 147.0 - 148.0 ppm for the quaternary carbons. The diphenylmethyl type displays very complicated signals in the region 126.0-129.5 ppm (for the CH sp^2 carbons) and near 139.4 ppm (for the Cq).

b) Other features, e.g., the less known heterocyclic systems (such as in cephamandole and ceftriaxone, Table 3.4) which required reference data to assign their carbon signals.

Table 3.4 : ^{13}C NMR characteristics of some cephalosporin antibiotics[a]. (continued overleaf)

(Formulae in Table 3.1, p.41)

Compound	Solvent Reference	Ring Carbons C-2 C-3 C-4 C-6 C-7	Carbonyls	R features (C-7 substituent)	R' features (C-3 substituent)
Cephalexin	D_2O-TFA HOD	30.3 137.9 122.1 58.0 57.1 (143) (182) (147)	169.8 (amido) 165.9 (CO_2H) 165.3 (lactam)	C-1' 132.3 3-Me 19.9 C-2' to 131.1,130.3, C-6' 128.8 C-7' 59.3 (157)	C-8'(Me) 19.9
Cefadroxil	CD_3OD-TFA TMS	30.9 135.5 124.4 60.1 61.3 (146) (180) (157)	170.5 (amido) 165.8 (CO_2H) 165.4 (lactam)	C-1' 123.6 C-2',6' 131.1 C-3',5' 117.3 C-4' 159.9 C-7' 57.6(145)	3-Me 20.3
Cefaclor	CD_3OD-TFA TMS	31.6 133.5 126.4 58.5 60.1 (149) (180) (157)	169.8 (amido) 164.5 (lactam) 163.8 (CO_2H)	C-1' 133.5 C-2' to 131.1,130.3 C-6' 129.2 C-7' 57.8(147)	
Cephalothin Na	D_2O HOD	26.0 116.8 132.3 57.9 59.7 (143) (181) (157)	173.8 (amido) 168.5 (CO_2Na) 165.1 (lactam) C-4' 126.0(190)	C-1' 136.3 C-2',C-3' 127.8 C-8'(Me) 20.8(131) C-5' 36.7(133)	C-6' 64.7(152) C-7' 174.2
Cefoxitin Na	D_2O HOD	25.9 118.0 131.7 63.4 95.4 (143) (182) (7-OMe, 53.7)	174.4 (amido) 168.2 (CO_2Na) 160.9 (lactam)	C-1' 135.9 C-2',C-3' 128.0,127.8 (171) C-4' 126.2(189) C-5' 36.7(132)	C-7' 64.5(154) C-8' 159.3

Cefuroxime Na	D_2O	26.0 117.4 131.9 57.7 59.4	168.5 (CO_2Na)	C-1' 144.8	C-7' 64.8(154)
	HOD	(145) (181) (159)	164.1 (amido)	C-2',C-3' 112.7,115.3	C-8' 159.4
			163.9 (lactam)	C-4'	146.6(214)
				C-5' 145.3	
				C-6'(Me) 63.2(147)	
Cefotaxime Na	D_2O	26.3 116.9 132.3 57.8 59.3	168.4 (CO_2Na)	C-1' 141.2	C-6' 64.7(152)
	HOD	(145) (179) (158)	164.8 (amido)	C-2' 113.3(195)	C-7' 174.3
			164.3 (lactam) C-3' 171.0	C-8'(Me) 20.8(133)	
				C-4' 148.4	
				C-5'(Me) 63.2(147)	
Cephamandole Li	D_2O	27.1 118.6 131.6 57.9 59.2	175.8 (amido)	C-1' 138.9	C-8' 36.9(150)
	HOD	(143) (179) (157)	168.0 (CO_2Na)	C-2' to 127.6	C-9 154.3
			164.7 (lactam)	C-6' 129.5	C-10(Me) 34.5
				C-7' 74.3(150)	
Cephazolin Na	D_2O	27.3 120.3 133.6 57.8 59.7	169.5 (CO_2Na)	C-1' 147.0(225)	C-3' 38.2(150)
	HOD	(144) (179) (157)	168.2 (amido)	C-2' 50.1	C-4' 168.9
			166.3 (lactam)		C-5' 171.7
					C-6'(Me) 14.9(134)

Table 3.5 : ^{13}C NMR features of some cephalosporin related compounds[a]

(Formulae in Table 3.3, p.64, and Chapter two, Table 2.2)

Compound	Solvent Reference	Ring Carbons C-2 C-3 C-4 C-6 C-7	Carbonyls	R features (C-7 substituent)	R' features (C-3 substituent)
7-ADCA	D_2O-TFA HOD	29.9 117.9 142.3 54.4 57.5	164.6 (CO_2O)) 160.7 (lactam)		3-Me 19.0
	D_2O-$NaHCO_3$ HOD	27.7 121.6 126.3 57.5 61.3 28.0 58.0 61.4	169.4 (CO_2H) 167.4 (lactam)		3-Me 18.2
Cephalothin lactone	DMSO-d_6 TMS 0.00ppm	22.5 122.8 142.8 57.4 59.8	170.1 (amido) 166.7 (lactone CO) 164.2 (lactam)	$ArCH_2$ 35.9 C-6' to 125.2,126.5 C-8' 126.8 C-5' 136.9	lactoneCH_2 71.5
2.8[b]	D_2O DSS 0.00ppm	28.4 122.2 127.0 56.9 58.8	175.5 (amido) 164.3 (CO_2Na) 161.0 (lactam)	$ArCH_2$ 42.0 C-4' 127.5 C-2',6' 129.3 C-3',5' 129.0 C_q-1' 134.7	3-Me 18.5
3.8[c]	CD_3OD TMS 0.00ppm	114.7 122.2 61.7 53.9 54.6	174.4 (amido) 170.9 (lactam 165.9 (CO_2H)	$ArCH_2$ 43.2 C-2' to 130.3, C-6' 129.5,128.0 C_q-1' 136.4	3-Me 22.3

a For abbreviation see footnotes under Table 3.4;

b 7-phenylacetamido-3-methyl-Δ^3-cephalosporin;

c 7-phenylacetamido-3-methyl-Δ^2-cephalosporin.

A few detailed assignments of ^{13}C spectra of some cephalosporin salts and esters follow. Examples have been selected in most cases from cephalosporins of recent introduction (e.g. cefixime ceftizoxime, ceftriaxone and cefsulodin). Coupled spectra were used for:

1) specific assignment of C-6 and C-7.

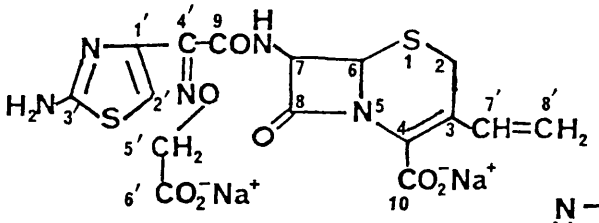
2) assignment of quaternary carbon (Cq) signals.

Relaxation time (T_1) measurements were used in a few examples to aid assignments of C-3 and C-4 and the carbonyl carbons.

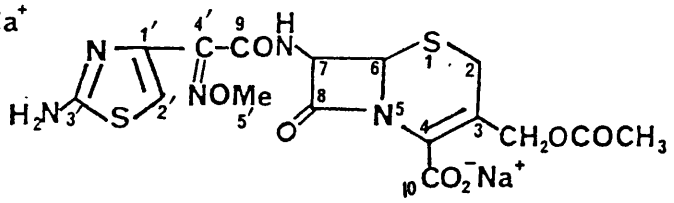
3.4.7 Detailed ^{13}C assignments of some cephalosporin salts

1. Cefixime Na

Assignments of ^{13}C signals was facilitated by published data on the close analogue. cefotaxime[84]:



Cefixime Na



cefotaxime Na

The proton decoupled ^{13}C NMR spectrum of cefixime in D_2O (solubilised with $NaHCO_3$) (Fig. 3.11) displayed the 12 low field (>100 ppm) and 4 high field signals required by the structure shown above. All signals could be assigned by reference to a coupled spectrum

(Fig. 3.12, p.79) and data for cefotaxime [Table 3.10, p.80].

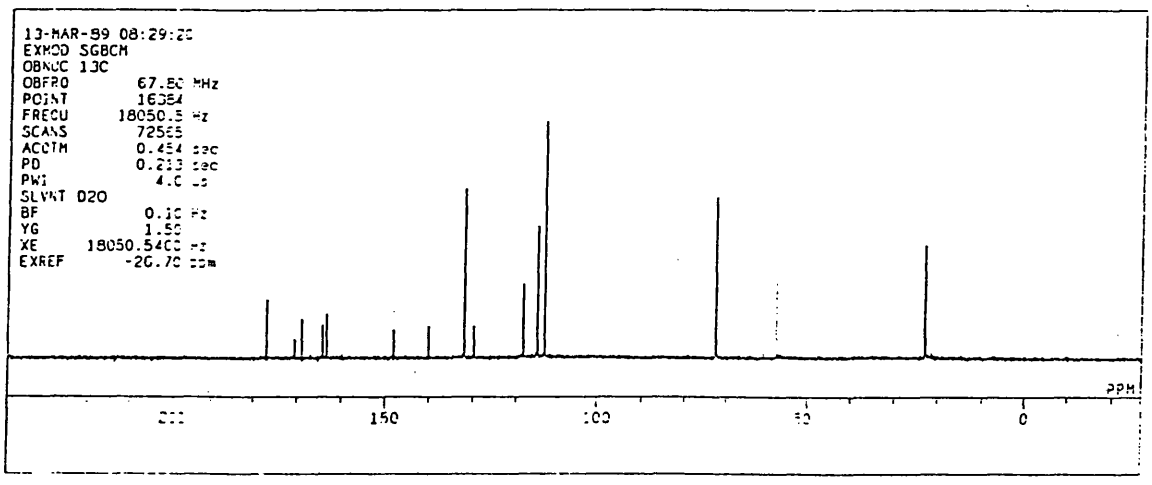
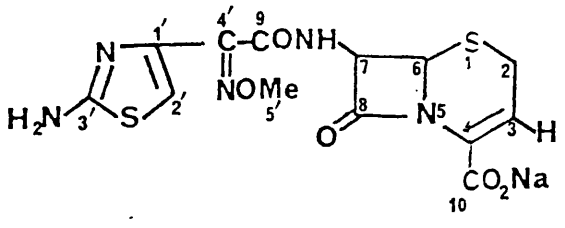


Fig. 3.11 : Proton noise-decoupled ^{13}C NMR spectrum (at 67.8 MHz) of cefixime Na in D_2O. (Experimental conditions as in section 3.3.1).

2. Ceftizoxime Na (in D_2O)



The data on cefotaxime are likewise of value for assignments of ^{13}C signals of ceftizoxime. The 13 carbon signals were well displayed in the proton decoupled spectrum (Fig. 3.13, p.82), 4 highfield of 100 ppm (due to $NO\underline{C}H_3$, C-7, C-6 and 2-$\underline{C}H_2$) and the rest lowfield. The ceftizoxime decoupled spectrum was also characterised by the low field signal of C_3-H (at 117.3 ppm) which is differentiated from the thienyl CH signal (at 113.1 ppm) from appearance of signals in coupled spectrum (Fig. 3.14, p.82); the former showed a

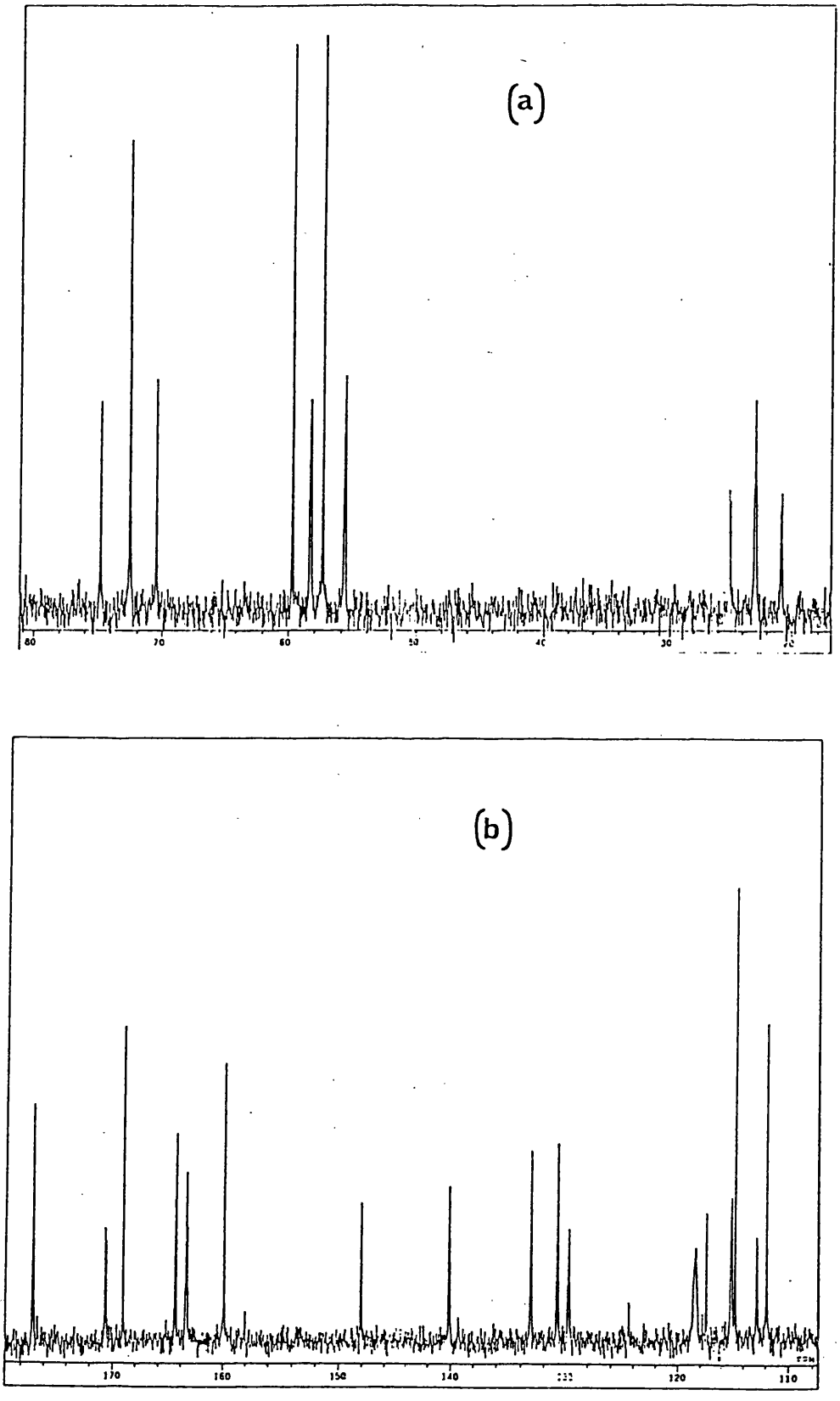


Fig.3.12 : Expansion of the ^{13}C-^{1}H NMR spectrum of cefixime Na in D_2O showing :
a) the high field (<100 ppm) and b) low field (>100 ppm) signal multiplicities.
See Table 3.10 for details.

Table 3.10 ^{13}C NMR characteristics of cefixime Na (in D_2O)*. (Formula p.77)

Chemical[a] shift	signal[b] appearance	assignment	Reference
177.0	nt	OCH_2CO_2H	$MeCO_2H$ 177.2(Stothers)[111]
170.6	nd	C-3'	C-3'171.0 (cefotaxime)[84]
169.1	s	4-CO_2Na	4-CO_2Na 168.4 (cefotaxime)[84]
164.4	nd	7-CONH	7-CONH 164.8 (cefotaxime)[84]
163.4	nt	lactam C=O	lactam 164.3 (cefotaxime)[84]
148.1	s	C-4'of 7-substituent	C-4' 148.4 (cefotaxime)[84]
140.2	nd	C-1' of 7-substituent	C-1' 141.2 (cefotaxime)[84]
132.0	Sd (155 Hz)	CH of 3-vinyl 2-propene	CH of 133.1 (Stothers)[111]
129.8	brs	C-4 of cephem nucleus	
118.5	brm	C-3 of cephem nucleus	
115.1	t (159)	CH_2 of 3-vinyl	CH_2 of 115.0 (Stothers)[111] 2-propene
113.3	sd (193)	C-2'of thiazole ring	C-2' 113.3 (195) (cefotaxime)[84]
72.7	St (159)	OCH_2CO_2H	
58.6	Sd (173)	C-7	
57.1	d(each line nd) (196)	C-6	
22.96	t (159) (+fine structure)	2-CH_2	

(N.B. signal at 160.0 ppm of coupled spectrum is an artifact).

* Chemical shifts to nearest 0.1 ppm, J_{CH} values (Hz) in parenthesis. S=sharp, br=broad, d=doublet, t=triplet, m=multiplet, n=narrow, s=singlet; a cefixime signals (ppm), b (from coupled spectra).

doublet of narrow triplets due to one bond coupling to hydogen (3-CH) and long range coupling to 2-CH_2, while the latter appeared as sharp doublet [$^1J_{(CH)}$ 191.7 Hz]. The complete ^{13}C signal assignments for ceftizoxime Na are listed in Table 3.11 (p.81).

Table 3.11 : Complete ^{13}C signal assignments for ceftizoxime Na (D_2O). (Formula p.78)

Chemical[a] shift	Signal appearance	Assignment	Reference
170.6	nd	C-3' of Thiazole ring	C-3' 171.0 (cefotaxime[84])
168.3	nd	4-CO_2Na	4-CO_2Na 168.4 (cefotaxime[84])
164.6	nd	7-amido C=O	7-amido 164.8 (cefotaxime[84])
164.0	nt	lactam C=O	lactam 164.3 (cefotaxime[84])
147.8	brs	Cq-4' of 7-substituent	Cq-4' 148.4 (cefotaxime[84])
140.1	nd	Cq-1' of 7-substituent	Cq-1' 141.2 (cefotaxime[84])
131.2	brm	C-4 of cephem nucleus	C-4 132.3 (cefotaxime[84])
117.3	d of nt	C-3 of cephem nucleus	C-3 116.9 (cefotaxime[84])
113.1	Sd (191.7)	C-2' of Thiazole ring	C-2' 113.3 (cefotaxime[84]) (195)
62.4	q (146.1)	OCH_3 (C-5')	OCH3 63.2 (147) (cefotaxime[84])
56.3	brd (176.3)	C-6 of cephem nucleus	C-6 57.8(179)(cefotaxime[84])
23.6	t of brm (143.2)	2-CH_2 of cephem nucleus	2-CH_2 26.3 (145) (cefotaxime[84])

(for the abbreviations see footnotes under cefixime)

a ceftizoxime signals (ppm)

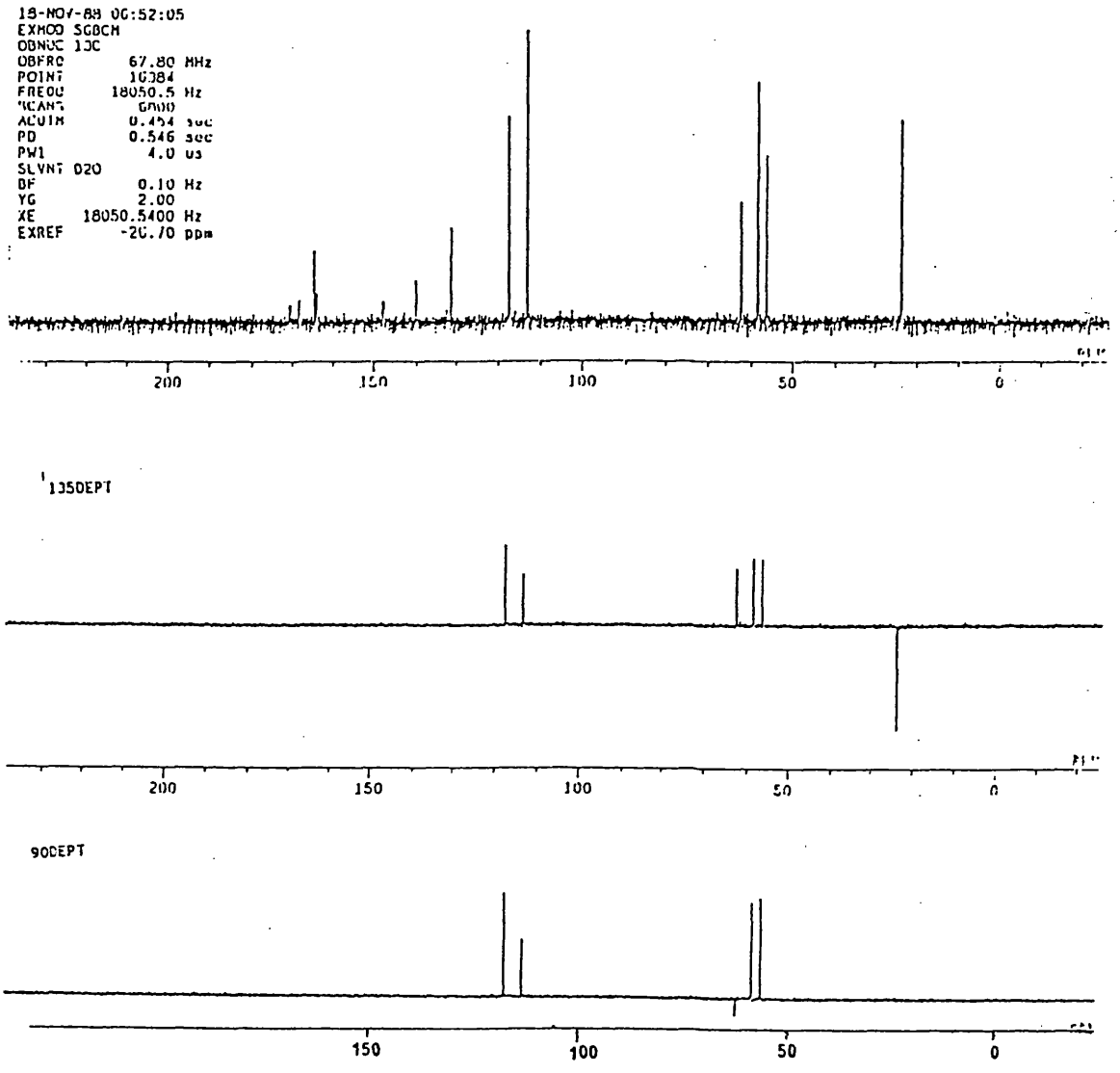


Fig. 3.13 : The 67.8 13C NMR proton noise decoupled (normal and DEPT) spectra of ceftizoxime Na in D_2O. (Experimental conditions as in section 3.3.1).

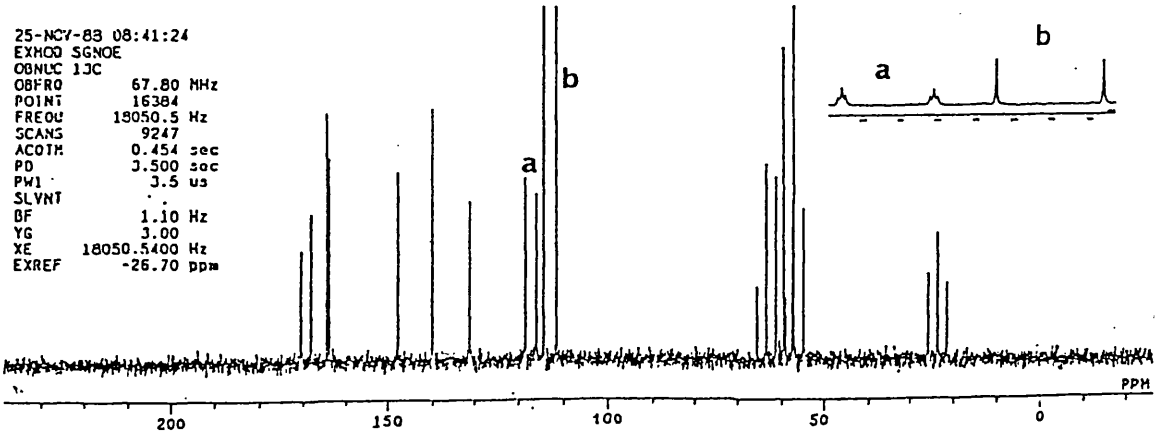
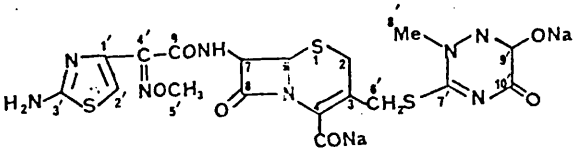


Fig. 3.14 : 13C-1H NMR coupled spectrum of ceftizoxime Na in D_2O. Insert : Expanded signals spectrum showing multiplicities due to C_3-H (at 117.3 ppm, narrow triplets) and thienyl CH (at 113 ppm as sharp doublet).

3. Ceftriaxone sodium (in D_2O)



The heterocyclic ring of the 3-substituent of ceftriaxone presents a problem for assigning carbons lowfield to 150 ppm; no reference data could be found to assign its carbon signals, and this task was achieved by considerations of carbon environments (expected chemical shifts behaviour). Other assignments were facilitated by the published cefotaxime data[84], a coupled spectrum (Fig. 3.15, p.83) and T_1 measurements (Fig. 3.16, p.84). Table 3.12 (p.85) lists the complete ^{13}C signal assignments for ceftriaxone Na. From the Table C-9' is the most deshielded compared to the other carbons of the triazine ring (resonating at 164.1 ppm as singlet); it is the less efficiently relaxed of these ring carbons[101], with the longer T_1 value (5.42 s). C-7' has more γ-hydrogens and resonates at higher field (156.3 ppm as a broad multiplet) with the shortest T_1 value (2.74 s). C-10' (the triazine ring carbonyl carbon) resonates between the two (at 160.4 ppm as singlet). C-3 and C-4 were assigned on basis of their multiplicities in the coupled spectrum and their T_1 values, C-3 (having 4 β-protons) more efficiently relaxed (T_1=1.41 s) than C-4 (T_1=3.48 s).

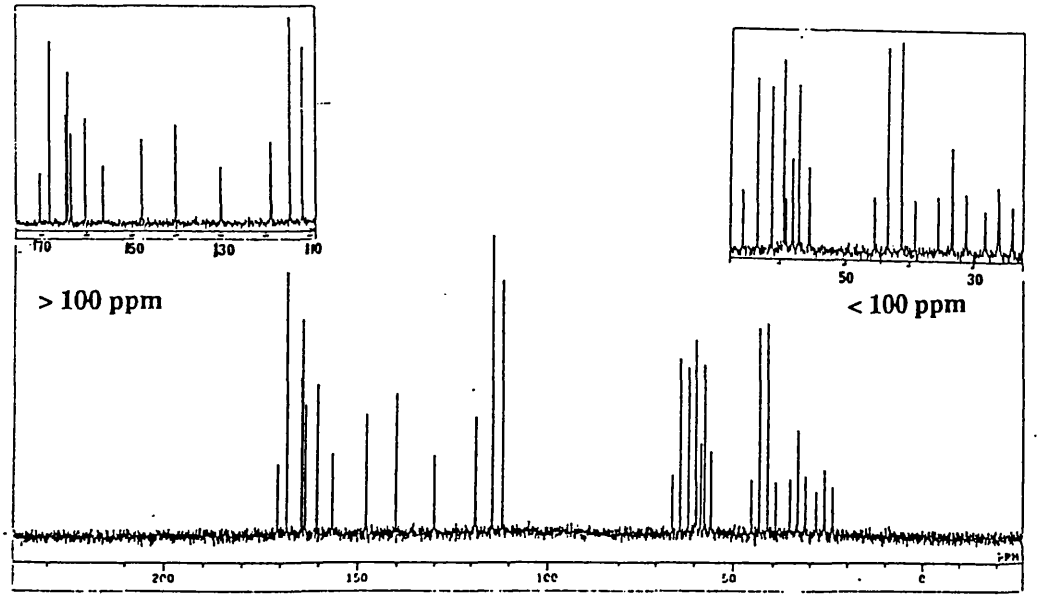


Fig. 3.15 : The 67.8 MHz 13C NMR coupled spectra, normal and expanded (inset) of ceftriaxone Na in D_2O. (Experimental conditions as in section 3.3.1).

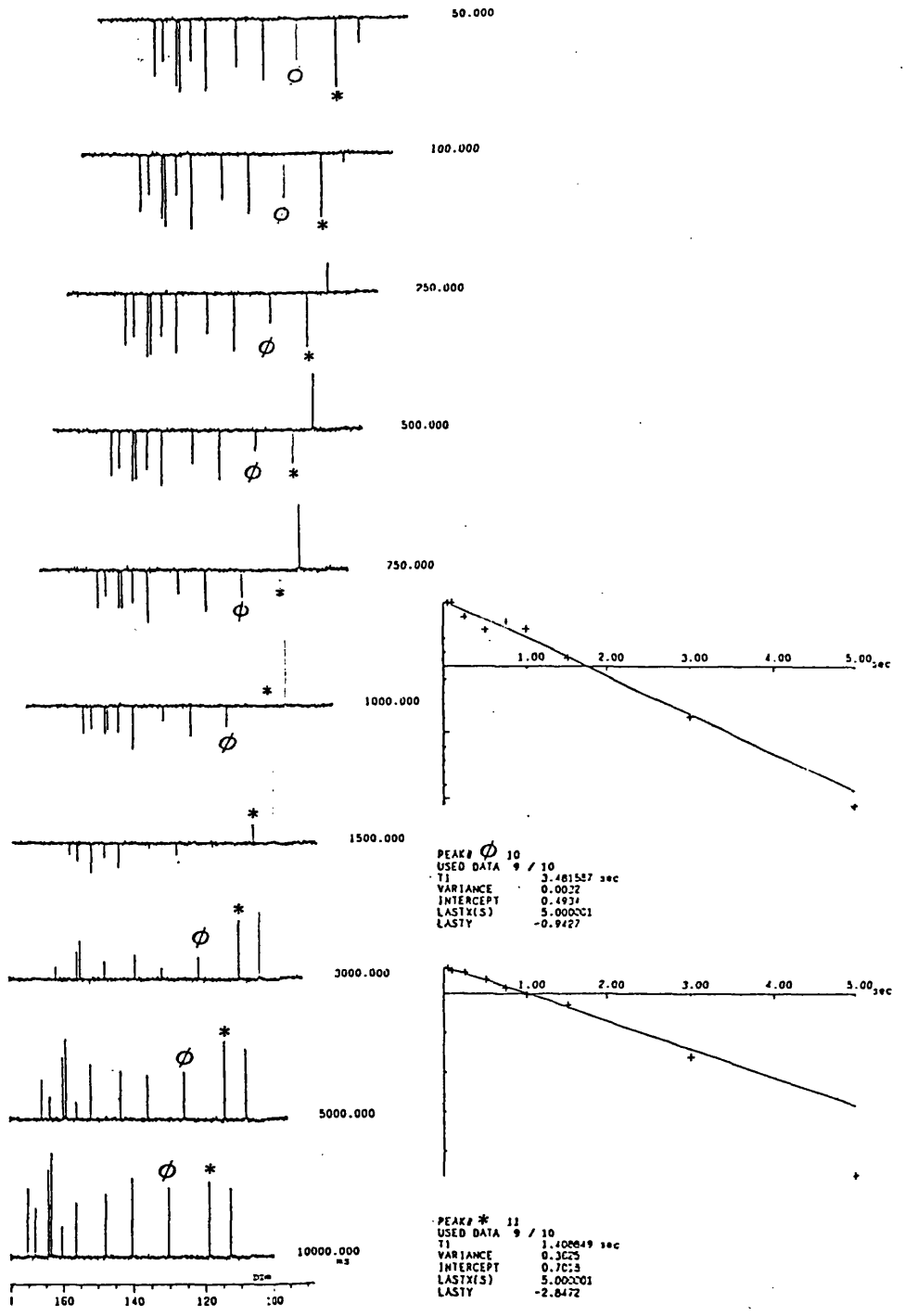


Fig 3.16 : a) Partial spin-lattice relaxation (T_1) ^{13}C NMR spectrum of ceftriaxone Na in D_2O showing C-3 (*) and C-4 (ϕ) signals, and b) linear regression plots for the calculation of C-3 and C-4 T_1 values (in sec).

(Experimental conditions as in section 3.3.1).

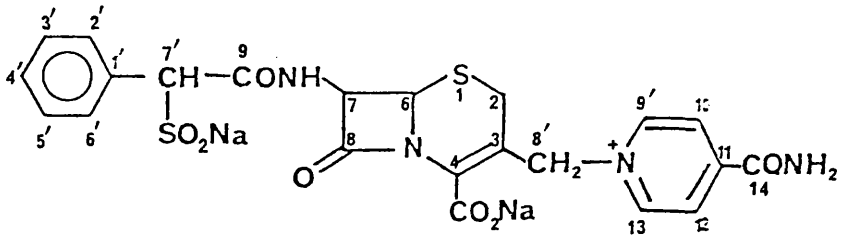
Table 3.12 : ^{13}C NMR characteristics of ceftriaxone Na (in D_2O). (Formula p.83)

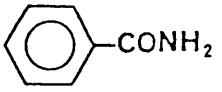
chemical[a] shift	signal appearance	assignment	Reference
170.4	nd	C-3' of thiazole ring	C-3' 171.0 (cefotaxime[84])
168.1	s	4-CO_2Na	4-CO_2Na 168.4 (cefotaxime[84])
164.4	nd	7-amido C=O (C-9)	7-amido 164.8 (cefotaxime[84])
164.1	s (T_1=5.4s)	C-9' of triazine ring	
163.4	nt	lactam C=O (C-8)	
160.4	s (T_1=3.38s)	triazine ring C=O (C-10')	
156.3	brs (T_1=2.74s)	C-7' of triazine ring	
147.8	s	C-4' of 7-substituent	C-4' 148.4 (cefotaxime[84])
140.3	nd	C-1' of 7-sustituent	C-1' 141.2 (cefotaxime[84])
130.2	brs (T_1=3.48s)	C-4 of cephem nucleus	
118.9	nt (T_1=1.41s)	C-3 of cephem nucleus	
112.9	d	C-2' of thiazole ring	C-2' 113.3 (cefotaxime[84])
62.5	q	OCH_3 (C-5')	OCH_3 63.2 (cefotaxime[84])
58.5	d (^1J=153)	C-7 of cephem nucleus	C-7 59.3 (cefotaxime[84])
57.1	d (^1J=175)	C-6 of cephem nucleus	C-6 57.8 (cefotaxime[84])
42.5	q	NMe (C-8')	
33.6	t	3-CH_2S	
26.4	t	2-CH_2 of cephem nucleus	2-CH_2 26.3(cefotaxime[84])

For the abbreviations see footnotes under cefixime.

a ceftriaxone signals (ppm).

4. Cefsulodin Na (in D_2O)





Benzamide

Assignments of ^{13}C signals was facilitated by some reference data of related compounds, cephaloridine[84] and benzamide[111], in addition to a coupled spectrum (Fig. 3.17 below). Data are given in Table 3.13 (p.87).

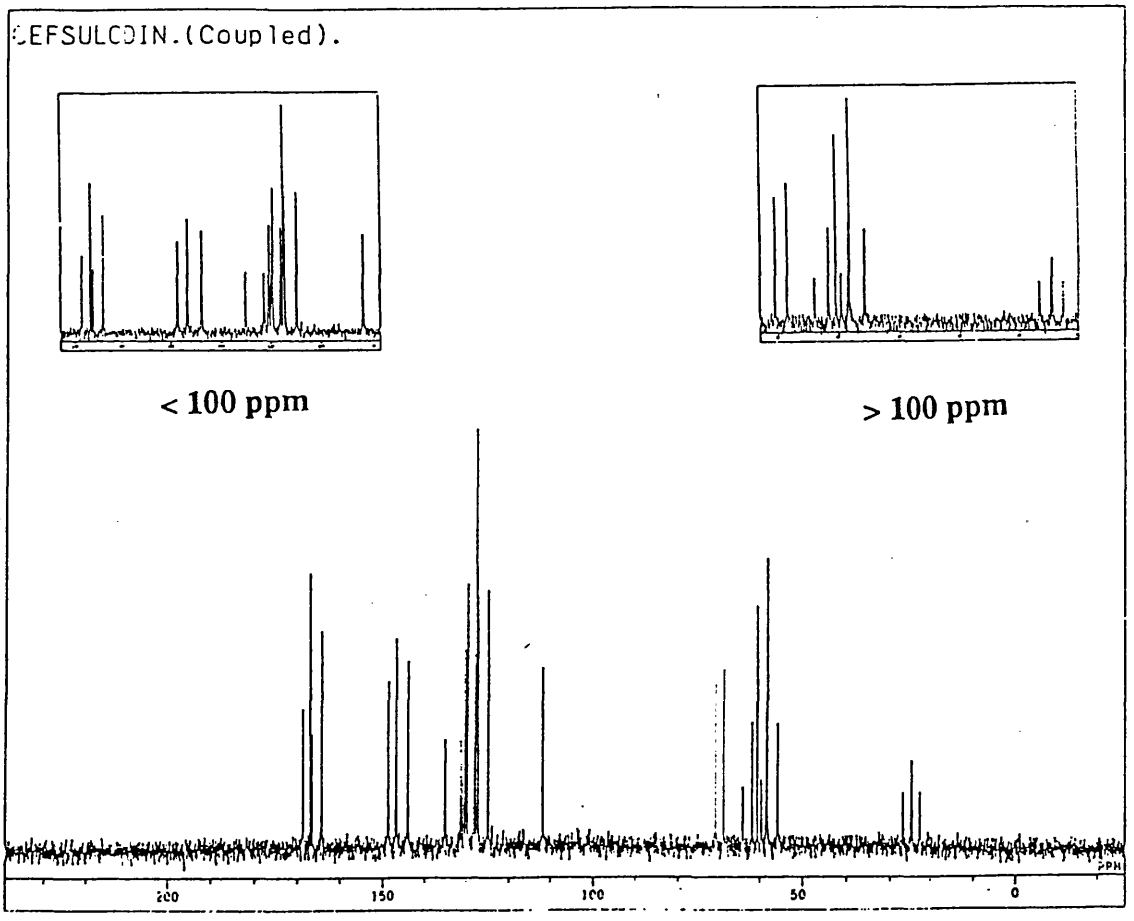


Fig. 3.17 : The 67.8 MHz ^{13}C NMR coupled spectra. normal and expanded (insert) of cefsulodin Na in D_2O. (Experimental conditions as in section 3.3.1).

Table 3.13 : ^{13}C NMR characteristics of cefsulodin Na (in D_2O). (Formula p.86)

Chemical[a] shift	signal appearance	assignment	Reference
168.6	nd	$CONH_2$ of 3-substituent (C-14)	PhCONH2 168.2 (benzamide[111])
166.8	Ss	4-CO_2Na	
166.4	brs	7-amido C=O (C-9)	
164.2	nt	lactam C=O (C-8)	
148.6	nt	Cq-11 of pyridyl ring	
145.2	brd	C-9',13 of pyridyl ring	C-7',11 145.2 (cephaloridine[84])
135.2	nm	C-4 of cephem ring	C-4 138.0 (cephaloridine[84])
131.4	nm	Cq-1' of phenyl nucleus	
129.2	d of nm	C-10',12 of pyridyl ring	C-8', 128.5 (cephaloridine[84]) 10'
128.7	d of nm	C-2' to	
128.4	d of nm	C-6' of phenyl ring	
126.2	d of nm		
111.8	nm	C-3 of cephem ring	C-3 110.0 (cephaloridine[84])
69.6	Sd	C-7' of 7-substituent	
61.8	t	3-CH_2S	3-CH_2S 62.4 (cephaloridine[84])
59.4	Sd (J=155)	C-7 of cephem nucleus	C-7 59.8 (cephaloridine[84])
57.2	d (each line nd)(J=176)	C-6 of cephem nucleus	C-6 57.6 (cephaloridine[84])
24.8	t of nm	2-CH_2 of cephem	2-CH2 24.6 (cephaloridine[84])

a cefsulodin signals (ppm); for other abbreviations see footnotes under cefixime.

3.4.8 Detailed ^{13}C assignments of cephalosporin esters

Interest

1) overall assignment of ^{13}C signals.

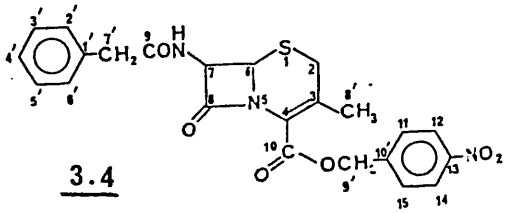
2) assignment of relative chemical shifts of C-3 and C-4.

As already mentioned under common carbon-13 spectral features of cephalosporins (p.67), literature observations and present results showed that for the alkali metal salts of cephalosporins C-3 resonates at higher field than C-4 (e.g., cephalothin Na : C-3 at 118.8 ppm, C-4 133.9 ppm[103], the former having the shorter T_1 (1.79 s) compared to 5.13 s of C-4); cephamandole lithium (present results) : C-3 118.6 ppm (T_1 2.8 s), C-4 131.6 ppm (6.0 s). In addition to our investigations on esters (this section), there is evidence from pH studies of cephaloridine[101] that C-3 becomes the lower field resonance when the 4-CO_2H group is non-ionised (low pH). T_1 data for cephalexin (free acid) are in support : T_1=2.54 s for 135.4 ppm resonance (C-3) and 4.36 s for that at 121.7 ppm (C-4).

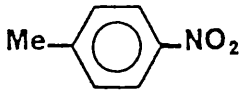
It was of interest therefore to investigate the relative chemical shifts of C-3 and C-4 of esters.

3.4.8.1 p-Nitrobenzyl type

1. 7-phenylacetamido-3-methyl-4-p-nitrobenzyl-Δ^3-cephalosporin ester 3.4 (in DMSO-d_6)



3.4



4-Nitrotoluene

Assignments of ^{13}C signals of 3.4 [Table 3.14, p.89] were based chiefly upon the expected chemical shift behaviour, DEPT technique (for those directly attached to hydrogen atoms) and on the separation values of a coupled spectrum. Furthermore, the correlation with published data of some references (such as benzylpenicillin, cephalexin, and 4-nitrotoluene) provided valuable aids to assignments . From the Table the signal at 132.6 ppm showed a

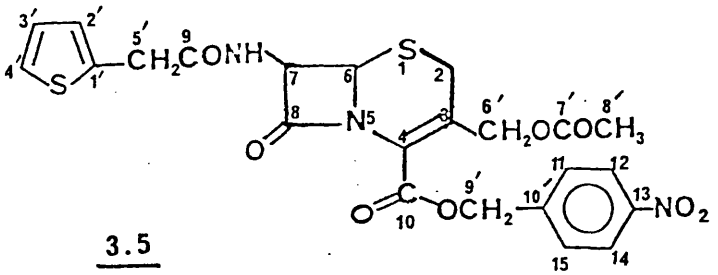
narrow quartet and was more intense than that at 121.2 ppm, so is assigned to C-3. The signal at 57.2 ppm appeared as a broad doublet in coupled spectrum with the larger separation [$^1J_{(CH)}$ 174.1 Hz] compared to that at 58.9 ppm which appeared as a sharp doublet and smaller separation [$^1J_{(CH)}$ 151.6 Hz]. Therefore the former signal is assigned to C-6 and the latter to C-7. It is also noted that 4-CO_2R resonates to higher field (161.6 ppm) than in ionised compounds (>164.0 ppm).

Table 3.14[a] : ^{13}C NMR characteristics of 7-phenylacetamido-4 — p-nitro-benzyldesacetoxycephalosporanoate ester 3.4 (in DMSO-d_6). (Formula p.88)

Chemical shift	signal appearance	assignment	Reference
170.9	brs (+fine structure)	7-amido C=O	7-amido 169.8 (cephalexin[84])
164.8	nt	lactam C=O	lactam 165.3 (cephalexin[84])
161.6	brs	4-CO_2R	
147.1	nt	Cq-13 of ester group	C-3 146.2 (4-nitrotoluene)
143.2	nm	Cq-10'of ester group	
135.7	nm	Cq-1' of 7-substituent	Cq-1' 134.8 (penicillin G)
132.6	nm	Cq-3 of cephem nucleus	
128.9	d of nm	C-11,15 of ester group	C-1, C-5 130.0 (4-nitrotoluene)
128.6	d of nm	C-2',6' of 7-substit.	C-2',6' 129.8 (penicillin G)
128.1	d of nm	C-3',5' of 7-substit.	C-3',5' 129.3 (penicillin G)
126.4	d of nm	C-4' of 7-substit.	C-4' 127.7 (penicillin G)
123.4	d of nm	C-12,14 of ester group	C-2,4 123.5 (4-nitrotoluene)
121.2	nm	Cq-4 of cephem nucleus	
65.3	t (151.5Hz)	CH_2 of ester group	CH2 60.1(Stothers[111])
58.9	sd (151.6)	C-7 of cephem nucleus	
57.2	brd (174.1)	C-6 of cephem nucleus	
41.5	t (131.0)	ArCH2 of 7-substit.	ArCH2 42.5 (133) (penicillin G)
29.1	t of nm	2-CH_2 of cephem nucleus	
19.5	brq	3-CH_3	3-Me 19.9 (cephalexin[84])

a For abbreviations see footnotes under Table 3.10;
7-substit.=7-($PhCH_2CONH$)

2. Cephalothin-4-p-nitrobenzyl ester 3.5 (in $CDCl_3$)



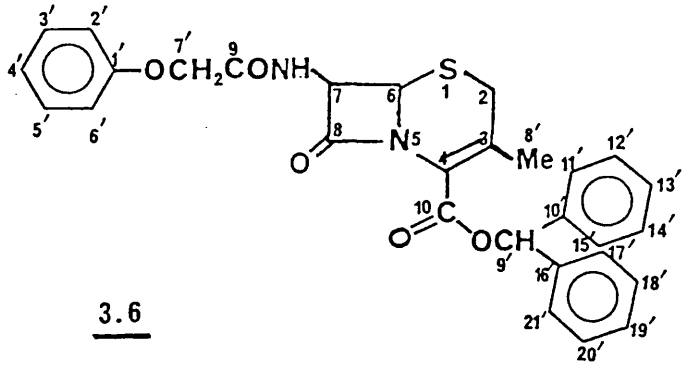
3.5

With reference to cephalothin Na (in D_2O) data and a coupled spectrum (Fig. 3.18, p.91), the assignments of the ^{13}C signals of 3.5 are shown in Table 3.15 (p.92).

It is noteworthy that the overlap arms of C-H signals in the aromatic region of the coupled spectrum (Fig. 3.18) presents a problem in the specific assignment of the aromatic carbons. The C-6 (57.3 ppm, 174 Hz) resonance is assigned to higher field of C-7 (59.3 ppm, 154 Hz) on basis of their $^1J_{(CH)}$ values. The C-3 signal was differentiated from C-4 from the intensities of their signals and multiplicities in coupled spectrum (in this case the C-3/ C-4 chemical shift difference was unusually small).

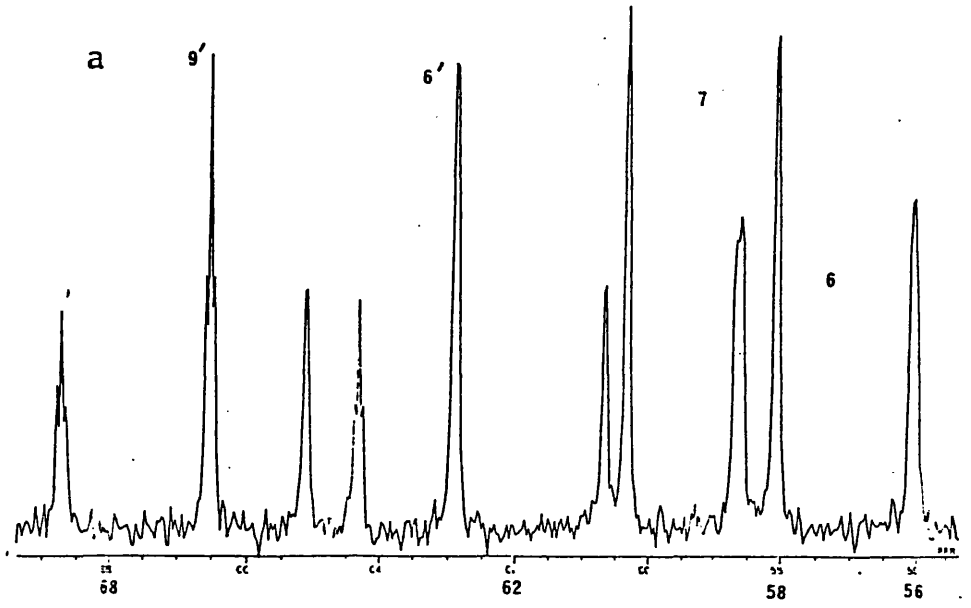
3.4.8.2 Diphenylacetate type

The example studied was the 7-phenoxyacetamido-3-methyl-4-diphenylacetate-Δ^3-cephalosporin ester 3.6 :



3.6

Due to the structural complexity of compound 3.6 (containing 29 carbons) several



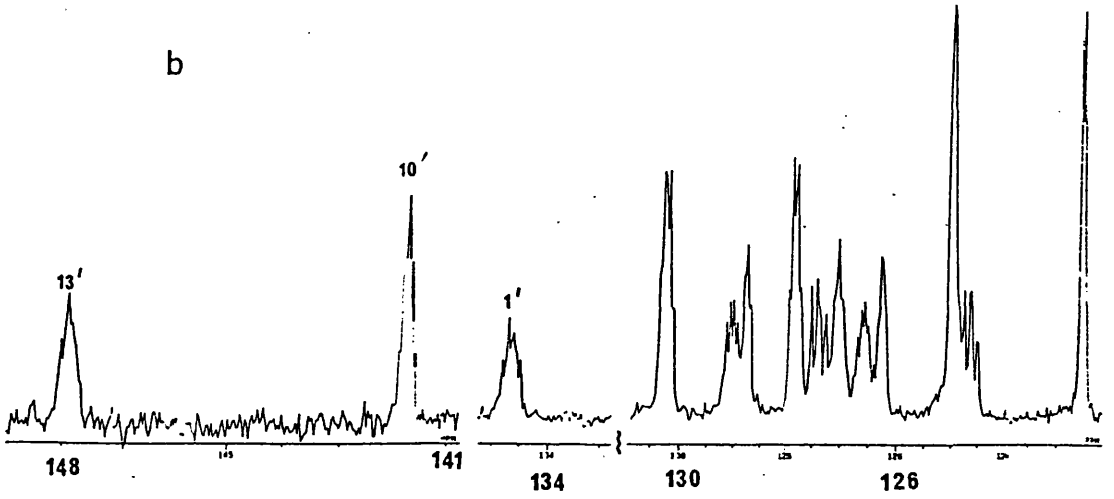


Fig. 3.18 : Expansions of the 67.8 MHz ^{13}C NMR coupled spectrum of 3.5 (in $CDCl_3$) showing a) the high field (<100 ppm) and b) low field signal multiplicities. See Table 3.14 for details.

Table 3.15 : ^{13}C NMR characteristics of cephalothin-4-p-nitrobenzyl ester 3.5 (in $CDCl_3$)[a]. (Formula p.90).

Chemical[b] shift	signal appearance	assignment	Reference
170.4	nm	OCOMe	OCOMe 174.2 (cephalothin Na)
169.9	nt	7-amido C=O	
164.8	nt	lactam C=O	lactam 165.1 (cephalothin Na)
160.9	brs	4-CO_2R	
148.0	nm	Cq-13 of e.gr.	Cq-3 146.2 (4-nitrotoluene)
141.8	nm	Cq-10' of e.gr.	
134.6	nm	Cq-1' of t.r.	Cq-1 136.3 (cephalothin Na)
129.1	d of nm	C-11,15 of e.gr.	C-1,5 130.0 (4-nitrotoluene)
127.9	d of nm	C-2',3' of	C-2,3 127.8 (cephalothin Na)
127.6	d of nm	thienyl ring	
127.0	brm	Cq-3 of c.n.	
126.1	d of nm	C-4' of t.r.	C-4 126.0 (cephalothin Na)
125.0	brs	Cq-4 of c.n.	
123.8	d of nm	C-12,14 of e.gr.	C-2,4 123.5 (4-nitrotoluene)
66.6	t of nt (150)	CH_2 of e.gr.	cf 1 65.3 (151.5)
62.9	t (each line brs) (152)	3-CH_2O	3-CH_2O 64.7 (cephalothin Na) (152)
59.3	Sd(154.2)	C-7 of c.n.	C-7 59.7 (157 (cephalothin Na)
57.3	d(each line brt) (174)	C-6 of c.n.	C-6 57.9 (cephalothin Na) (181)
37.1	t (130)	CH^2 of 7-substituent	CH_2 36.7 (cephalothin Na) (133)
26.4	t of brm (142)	2-CH_2 of cephem nucleus	2-CH_2 26.0 (cephalothin) (143)
20.7	q (129.3)	CH_3 of 3-substituent	CH_3 20.8 (cephalothin Na) (131)

a For abbreviations see footnotes under Table 3.10; e.gr.=ester group, t.r.=thienyl ring, c.n.=cephem ring. b 3.5 signals in ppm.

reference compounds (such as phenoxymethyl penicillin Na, benadryl HCl), in addition to a coupled spectrum (Fig. 3.19, p.95) and T_1 measurements (Fig. 3.20, p.96), were employed for the assignment of the various ^{13}C signals (Table 3.16).

Table 3.16 : ^{13}C NMR characteristics of 3.6 (in $CDCl_3$)[a]. (Formula p.90).

Chemical[a] shift	signal appearance	assignment	Reference
168.5	m (T_1=4.36s)	7-amido C=O	
164.0	nt (4.73s)	lactam C=O	
161.0	brs (4.67s)	4-CO_2R	
156.8	nm (10.4s)	Cq-1' of 7-sub.	C-1' 155.6 (Stothers[111])
139.4	m (4.98s)	Cq-10',16' of d.p.a.	C-2,8 140.9 (benadryl)
139.3	m (2.91s)		
134.0	nm (8.06s) (6 lines)	Cq-3 of c.n.	
129.5	d of nm	C-3',5' of 7-sub.	C-3',5' 130.5 (ph.m.pen.)
128.3	d of nt	C-13',19' of d.p.a.	C-5,11 128.7 (benadryl)
128.2	d of nt	C-13',19' of d.p.a.	
127.8	d of nm	C-11',15',17',21' of d.p.a.	C-3,7, 127.9 (benadryl) 9,12
127.0	d of nm	C-12',14',18',20'	C-4,6, 126.6 (benadryl)
126.8	d of nm	of d.p.a.	C-3',5' 130.5 (ph.m.pen.)
122.4	m (6 lines) (5.14sec)	Cq-4 of cephem nucleus	
122.0	d of nt (0.83sec)	C-4' of 7-sub.	C-4' 122.7 (ph.m.pen.)
114.6	d of nm	C-2',6' of 7-sub.	C-2',6' 115.2 (ph.m.pen.)

78.7	d(each line brs)(147.7Hz)	$CHPh_2$ (C-9')	$CHPh_2$ 83.7 (benadryl)
58.1	d(154)(T_1=0.72s)	C-7 of c.n.	
56.9	brd(174) (0.71s)	C-6 of c.n.	
30.1	t of nm(144) (0.31s)	2-CH_2 of cephem nucleus	
19.9	Sq(129)(1.55s)	3-CH_3	

a For abbreviations see footnotes under Table 3.15; 7-sub.=7-substituent, d.p.a.=diphenylacetate group, ph.m.pen.=phenoxymethylpenicillin. c.n=cephem nucleus

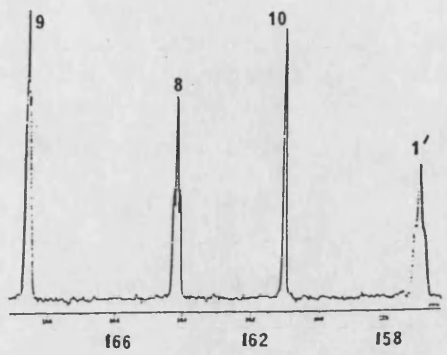
b 3.6 signals in ppm.

In brief, the following was observed from the Table :

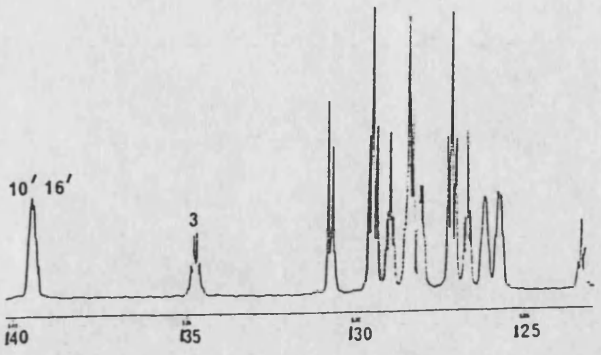
a) The T_1 data of C-3 and C-4 did not, for unknown reasons, correlate with their relative assignments, i.e., assignment of signal at 134 ppm to C-3 was not supported by relative T_1 value (8.06 s) compared to that of C-4 at 122.4 ppm (with T_1=5.14 s). The 134 ppm signal showed more couplings than that at 122.4 ppm and was therefore assigned to C-3.

b) Using T_1 values to differentiate between the three carbonyl carbons (amido, lactam, and 4-CO_2R) (T_1 of Cq decreases with an increasing number of α and β hydrogens[104]); thus, 7-amido has two β hydrogens, 8-CO lactam one, and 4-CO_2R none; therefore the signal at 168.5 ppm with the shortest T_1 value is assigned to 7-amido carbonyl carbon, that at 164.0 ppm and 161.0 ppm have close T_1 values, but,the former appearing as narrow triplet in the coupled spectrum is assigned to the lactam, and that at 161.0 ppm (a broad singlet) to 4-CO_2R.

c) The T_1 value (0.83 s) of the signal at 122.0 ppm compared to that at 122.4 ppm (5.14 s) clearly confirm assignment of the higher field resonance to a CH carbon.



Expansion of 170-155 ppm region



Expansion of 140-120 ppm region

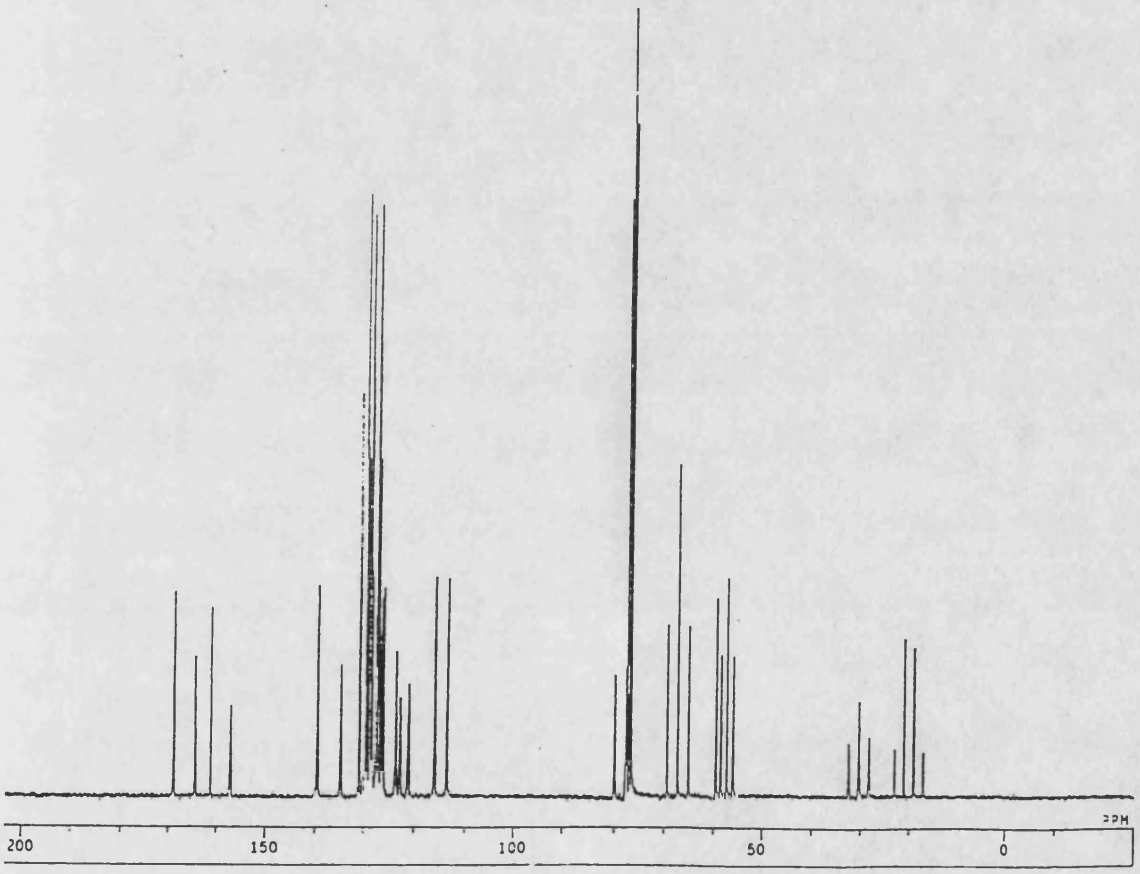


Fig. 3.19 : The 67.8 MHz ^{13}C NMR coupled spectrum of 3.6 in $CDCl_3$. (Experimental conditions as in section 3.3.1)

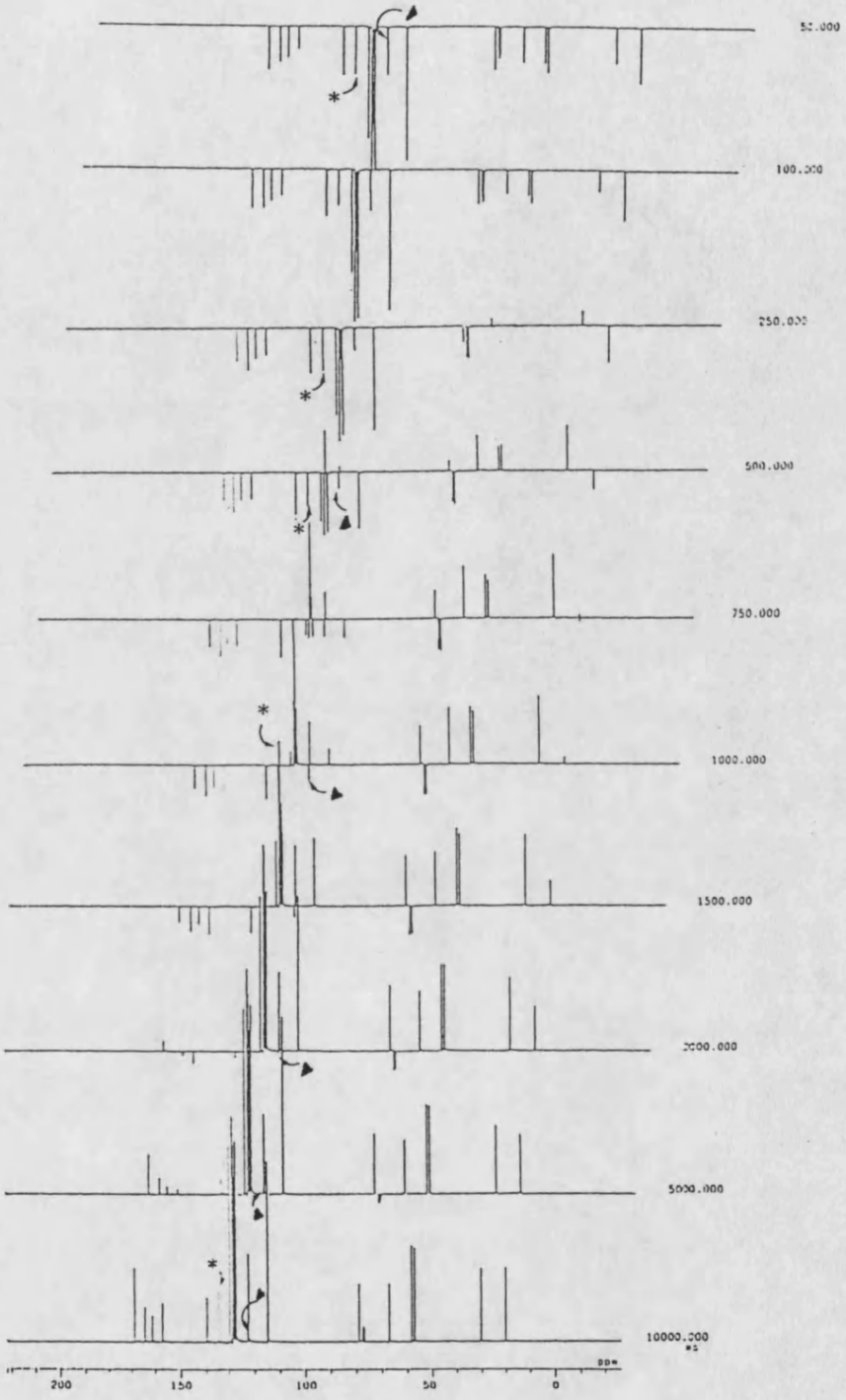
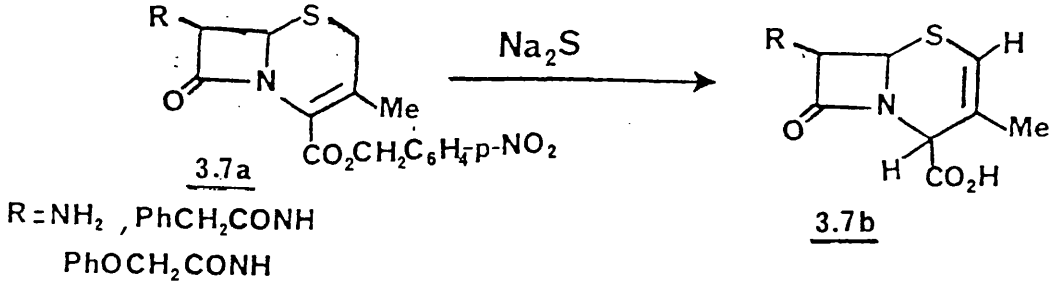


Fig. 3.20 : Spin-lattice relaxation (T_1) ^{13}C NMR spectrum of 3.6 in $CDCl_3$. Arrows indicate C-3 (*) and C-4 (▲) signals. (Experimental conditions as in section 3.3.1).

3.4.9 Ceph-2-ene work

While searching the literature for methods for the removal of the p-nitrobenzyl acid protecting group a paper by a Lilly research group was found which described the use of sodium sulphide (Lammert et al., 1978)[89]. The products resulting from treatment of the cephalosporin esters 3.7a proved however to be corresponding to 2-cephem acids 3.7b rather than 3-cephem derivatives :

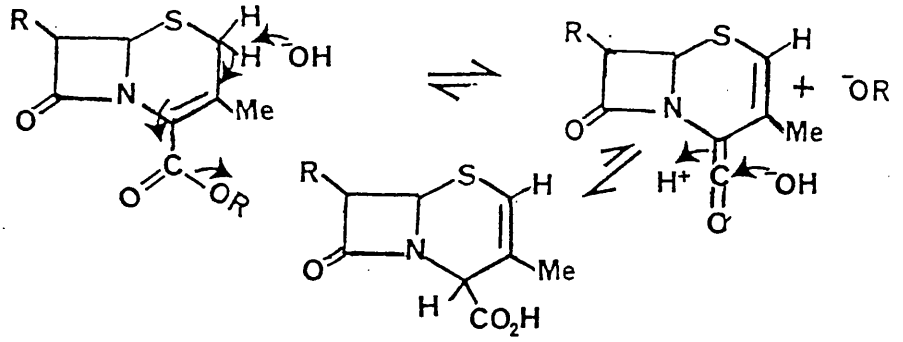


3.7a

R = NH_2 , $PhCH_2CONH$
$PhOCH_2CONH$

3.7b

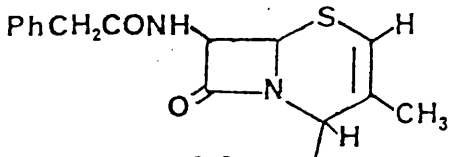
Since the reaction is carried out under alkaline conditions double bond migration is probably base catalysed, as shown :



Conversion of ceph-3-enes (i.e., Δ^3-cephalosporins) to ceph-2-enes have in fact been reported by treatment of the antibiotics with aquecus pyridine and 1N NaOH (Van Heyningen et al., 1968)[34].

The possibility of ceph-2-enes forming as one of the components of degraded cephalosporin antibiotics has not been investigated. For this reason, it was considered of interest to prepare authentic ceph-2-ene derivatives for use as reference standards in NMR stability investigations.

3.4.9.1 Synthesis of ceph-2-ene 3.8



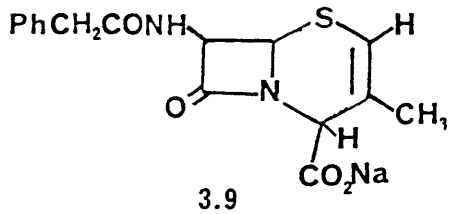
3.8

7-phenylacetamido-3-methyl-2-cephem-4-carboxylate 3.8 was synthesised in our laboratories according to the method of Lammert et al.[89] as described in Chapter two (section 2.2.3.6).

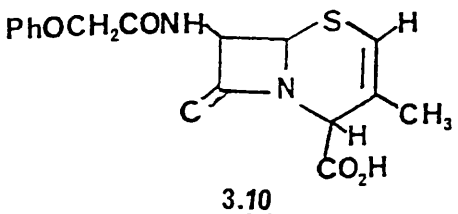
3.4.9.2 Characterisation of 3.8

a) ^{1}H NMR

Assignments of the proton signals of 3.8 (Fig. 3.21, p.100) was facilitated by published data on: the sodium salt of 7-phenylacetamido-3-methyl-Δ^2-cephalosporin 3.9 (Mondelli et al.[110]) and 7-phenoxymethyl acetamido-3-methyl-2-cephem-4-carboxylic acid 3.10 (Lammert et al.[89]):



3.9



3.10

Table 3.17 : ^{1}H NMR characteristics of 3.8 (in $CDCl_3$ + DMSO-d_6)[a]. (Formula p.98).

Chemical[b] shift	signal appearance	assignment	Reference (3.10 in $CDCl_3$+DMSO-d_6)
8.83	d (8.06)	7-NH	NH 7.90 d (9.0)
7.24-7.31	m	Ph (5H)	Ph 6.86-7.48 m
6.00	nm	2-H	(2-H not reported)
5.48	dd (3.84, 8.06)	7-H	7-H 5.64 dd (5.0, 9.0)
5.20	d (4.0)	6-H	6-H 5.35 d (5.0)
4.65	brs	4-H	4-H 4.67 brs
3.59	dd	Ar-CH_2	OCH_2 4.60 s
1.94	brs	3-CH_3	3-CH_3 1.98 s

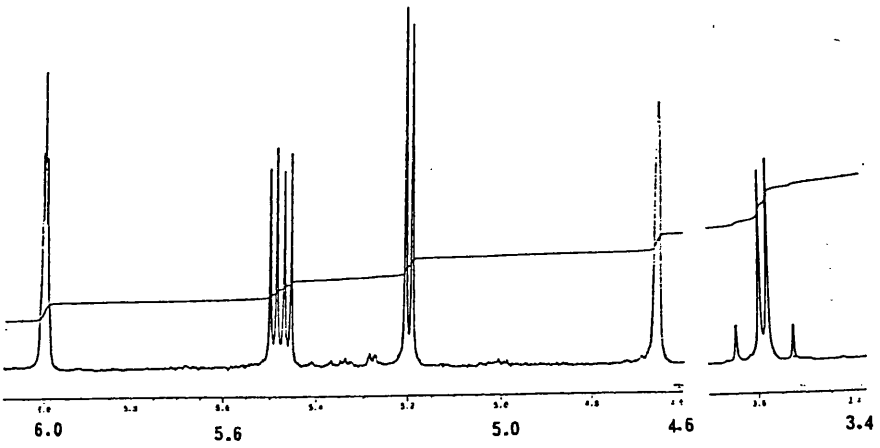
a m=multiplet, nm=narrow multiplet, d=doublet, dd=doublet doublets brs=broad singlet, Ph=Phenyl, Ar=Aromatic. J values in Hz in parenthesis.

b 3.8 signals in ppm from TMS.

Table 3.18 : ^{1}H NMR characteristics of 3.8 (+$NaHCO_3$ in D_2O)[a]. (Formula p.98).

Chemical[b] shift	signal appearance	assignment	Reference (3.9 in D_2O)
7.33-7.40	m	Ph (5H)	no report
5.95	nm	2-H	no report
5.38	d (3.9)	7-H	7-H 5.40 d (4.0)
5.25	d (3.9)	6-H	6-H 5.27 d (4.0)
4.57	brs	4-H	4-H 4.60 s
3.69	dd	Ar-CH_2	Ar-CH_2 3.66 brs
1.86	brs	3-CH_3	

a For abbreviations see footnotes under Table 3.17; b 3.8 signals (ppm)



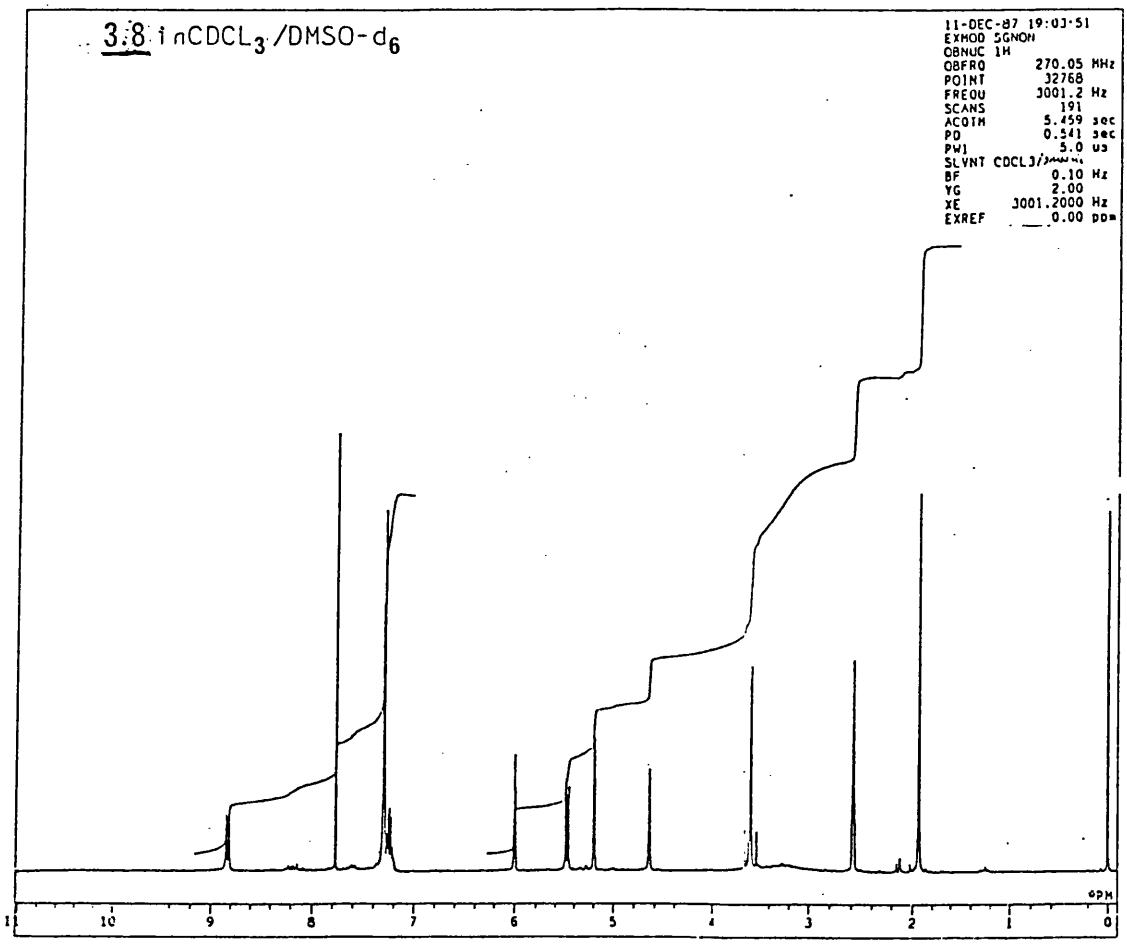


Fig. 3.21 : 270 MHz ^{1}H NMR spectrum of 3.8 in $CDCl_3$ + DMSO-d_6. (Experimental conditions as in section 3.3.1).

b) ^{13}C NMR

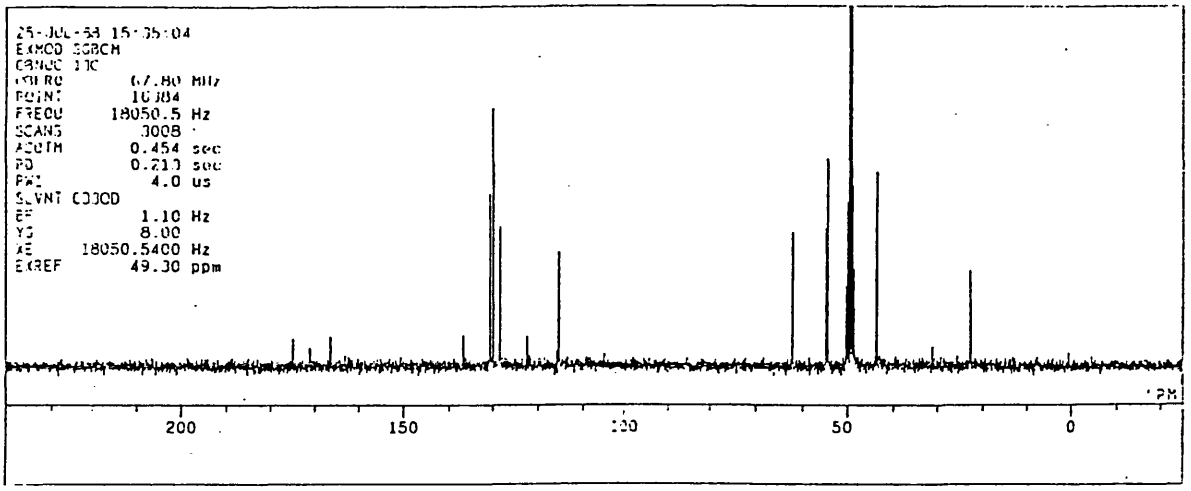
The ^{13}C data on 3.9 are likewise of value for the assignments of ^{13}C signals of 3.8 (Fig. 3.22, p.102) as follows :

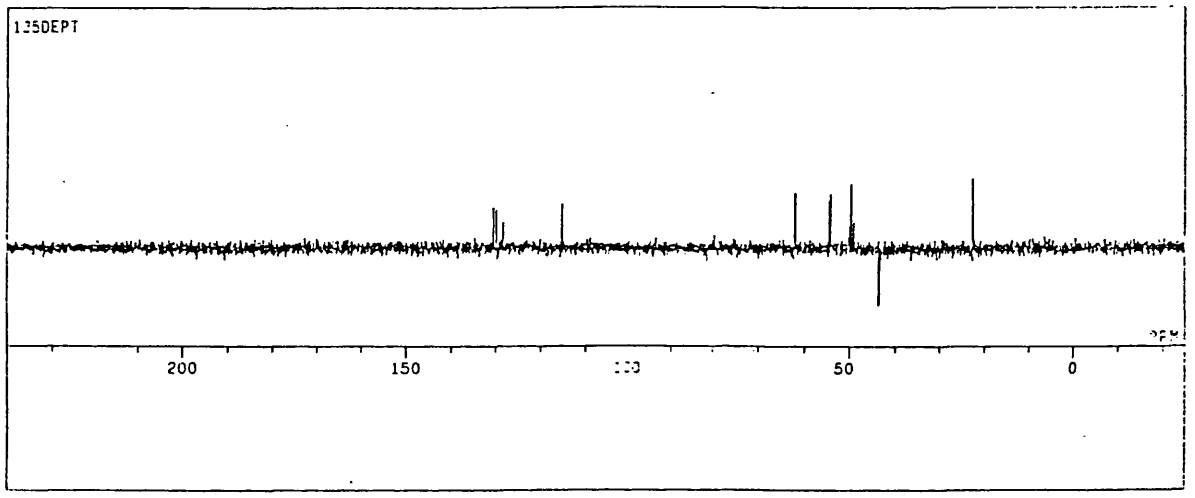
Table 3.19 : ^{13}C NMR characteristics of 3.8 (in CD_3OD)[a]. (Formula p.98).

Chemical[b] shift	assignment[c]	Reference[c] (3.9 in D_2O)
174.4	7-amido C=O	7-CONH 176.0
170.9	lactam C=O	lactam 166.4
165.9	4-CO_2H	4-CO_2Na 175.2 (unusually lowfield)
136.4	Cq-1' of Ph ring	Cq-1' 135.5
130.3	C-2' to	C-2' to 130.3
129.5	C-6' of	C-6' of 129.9
128.0	Ph ring	Ar ring 128.4
122.2	Cq-3 of c.n.	Cq-3 126.4
114.7	C-2 of c.n.	C-2 111.8
61.7	C-4 of c.n.	C-4 57.4
54.6	C-7 of c.n.	C-7 60.4
53.9	C-6 of c.n.	C-6 53.4
43.2	Ar-CH_2	Ar-CH_2 42.8
22.3	3-Me	3-Me 22.2

a shifts are in ppm from TMS; b 3.8 signals (ppm);

c Ph=Phenyl, Ar=Aromatic, c.n.=cephem nucleus.





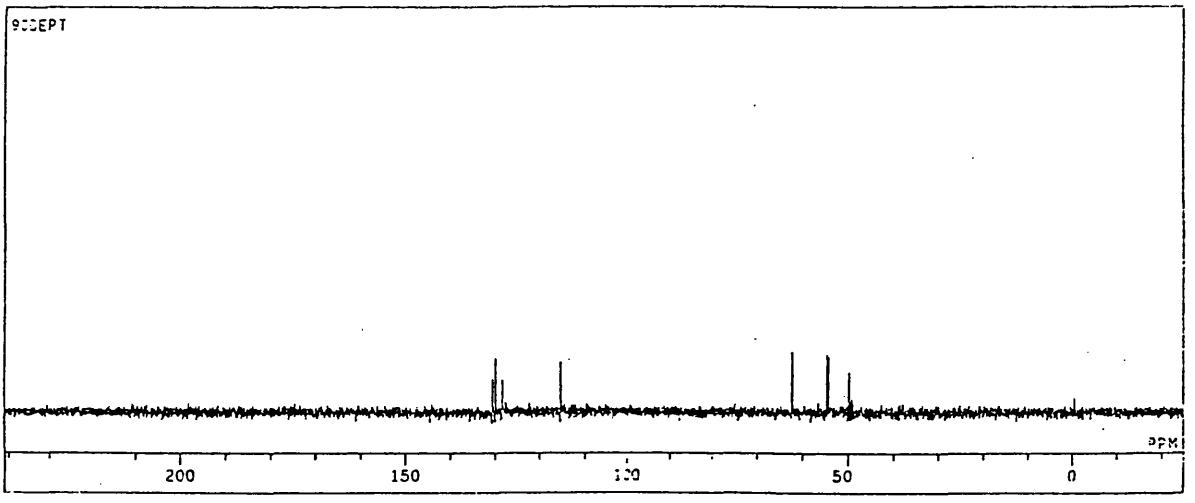
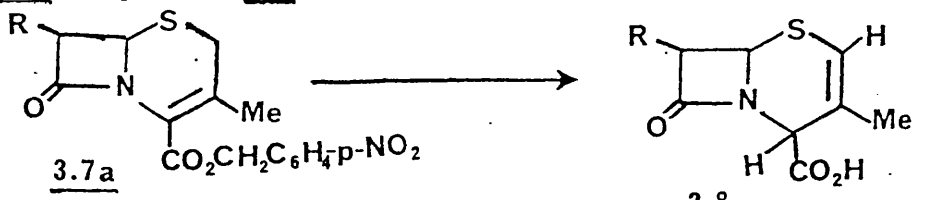


Fig. 3.22 : 67.8 MHz ^{13}C NMR proton noise-decoupled spectra (normal and DEPT) of 3.8 in CD_3OD. (Experimental conditions as in section 3.3.1).

3.4.9.3 ^{1}H NMR evidence of interconversion of 7-phenylacetamido derivative of 3.7a to ceph-2-ene 3.8



R = $PhCH_2CONH$

Table 3.20 : ^{1}H NMR assignments[a] of 3.7a and 3.8

3.7a (in DMSO-d_6)			3.8 (in $CDCl_3$+DMSO-d_6)		
Chemical shift	signal appearance	assignment	chemical shift	signal appearance	assignment
9.12	d (8.2)	NH	8.83	d (8.1)	NH
8.24	d (8.8)	ArH-10',11'			
7.69	d (8.6)	ArH-9', 12'			
7.25-7.31	m	Ph (5H)	7.24-7.31	m	Ph (5H)
5.68	dd (4.6, 8.2)	7-H	5.48	dd (3.84, 8.1)	7-H
			6.00	nm	2-H
5.40	s	CH_2 of ester group			
5.09	d (4.8)	6-H	5.20	d (4.03)	6-H
			4.65	brs	4-H
3.54	d (8.4)	CH_2 of 7-substituent	3.59	dd	CH_2 of 7-substituent
3.45,3.63	d (18.4)	2-CH_2			
2.06	s	3-Me	1.94	brs	3-Me

a for abbreviation see footnotes under Table 3.17.

^{1}H NMR evidence of 3.7a interconversion to 3.8 is provided by :

1- Absence of 2-CH_2 four line AB signal.

2- lowfield singlet (at ~ 6.0 ppm) assigned to vinylic proton at C-2.

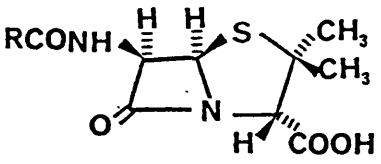
3- new singlet (near 4.7 ppm) assigned to methine proton at C-4.

3.4.9.4 NMR evidence of the configuration of ceph-2-enes 3.8 (at C-4)

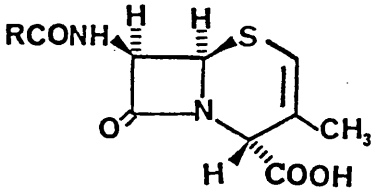
Off-resonance proton decoupling and ^1H-^1H COSY experiments were employed to aid solving the problem of configuration of the ceph-2-ene 3.8 at C-4.

It has been shown that the Δ^2-cephalosporins (compared to Δ^3) were almost completely inactive as antibiotics. This was attributed to an unreactive β-lactam system[112]. Another possible factor of the inactivity of Δ^2-cephalosporins must also be considered, and that is the absolute configuration of the 4-carboxyl substituent.

Van Heyningen et al.[34] have shown (by chemical correlation, see below) that the carboxyls in penicillins (C-3) and Δ^2 cephalosporins (C-4) possess the same absolute configurations (Fig. 3.23), and concluded that it is not the 4-carboxyl configuration but β-lactam stability that explains the feeble biological activity of Δ^2-cephalosporin.



A penicillin

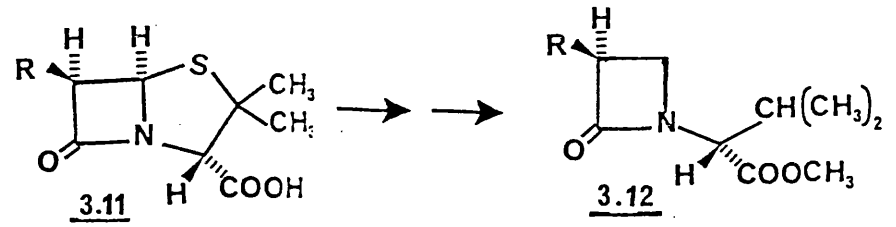


A Δ^2-cephalosporin

Fig. 3.23 : Configuration of the carboxy group in penicillins (C-3) and Δ^2-cephalosporins (C-4).

The plan of the authors[34] was to desulphurize phenoxymethylpenicillin 3.11 and obtain, after esterification, phenoxymethyl desthiopenicillin methyl ester 3.12. Similarly, they hydrogenated 7-phenoxyacetamido — Δ^2 — desacetoxy — cephalosporanic acid to a tetrahydrothiazine and desulphurized it to 3.12, which possess identical NMR, IR, and UV spectra as that obtained from 3.11. Furthermore, their X-ray powder diagrams coincide exactly. The authors subjected 3.12, from Δ^2-cephalosporin, to acid hydrolysis and isolated valine. Although its rotation was low ([α] -245 °) compared to pure D-valine (-563 °), the

rotation was strongly negative like D-valine (i.e. the product was predominantly the D-configuration). This proved that the carboxyls in penicillins and Δ^2-cephalosporins possess the same absolute configuration.



R = $PhOCH_2CONH-$

The purpose of the present work was to confirm (or otherwise) the above configuration at C-4 by NMR techniques (proton decoupling and COSY) i.e., by physical methods that avoid vigorous chemical reactions which may lead to stereochemical change.

1. ^1H-^1H 2D COSY experiment (of 3.8 in $CDCl_3$ + DMSO-d_6)

The spectrum (Fig. 3.24, p.106) was recorded on a Jeol JNM-GX 270 spectrometer, operating at 20.0°C, at a frequency of 3090.2 Hz, giving a digital resolution of 12.07 Hz. A 14.0 μs pulse (PW1) was employed with a 0.083 sec acquisition time and a 1.00 sec pulse delay between pulses.

The diagonal points in the spectrum showed where the like signals meet, while the off-peak points indicate the coupled signals. The more intense the points the stronger the coupling. The low-field NH (at 8.83 ppm) showed strong coupling to the 4-line signal at 5.48 ppm (due to 7-H). The 2-H signal at 6.00 ppm showed very clear coupling to the 3-Me signal at 1.94 ppm, and to a lesser extent (but clear) to the signal at 4.65 ppm (due to 4-H). Also the 4-H signal (at 4.65 ppm) showed similar long range couplings to both 3-Me (at 1.94 ppm) and 2-H (at 6.0 ppm).

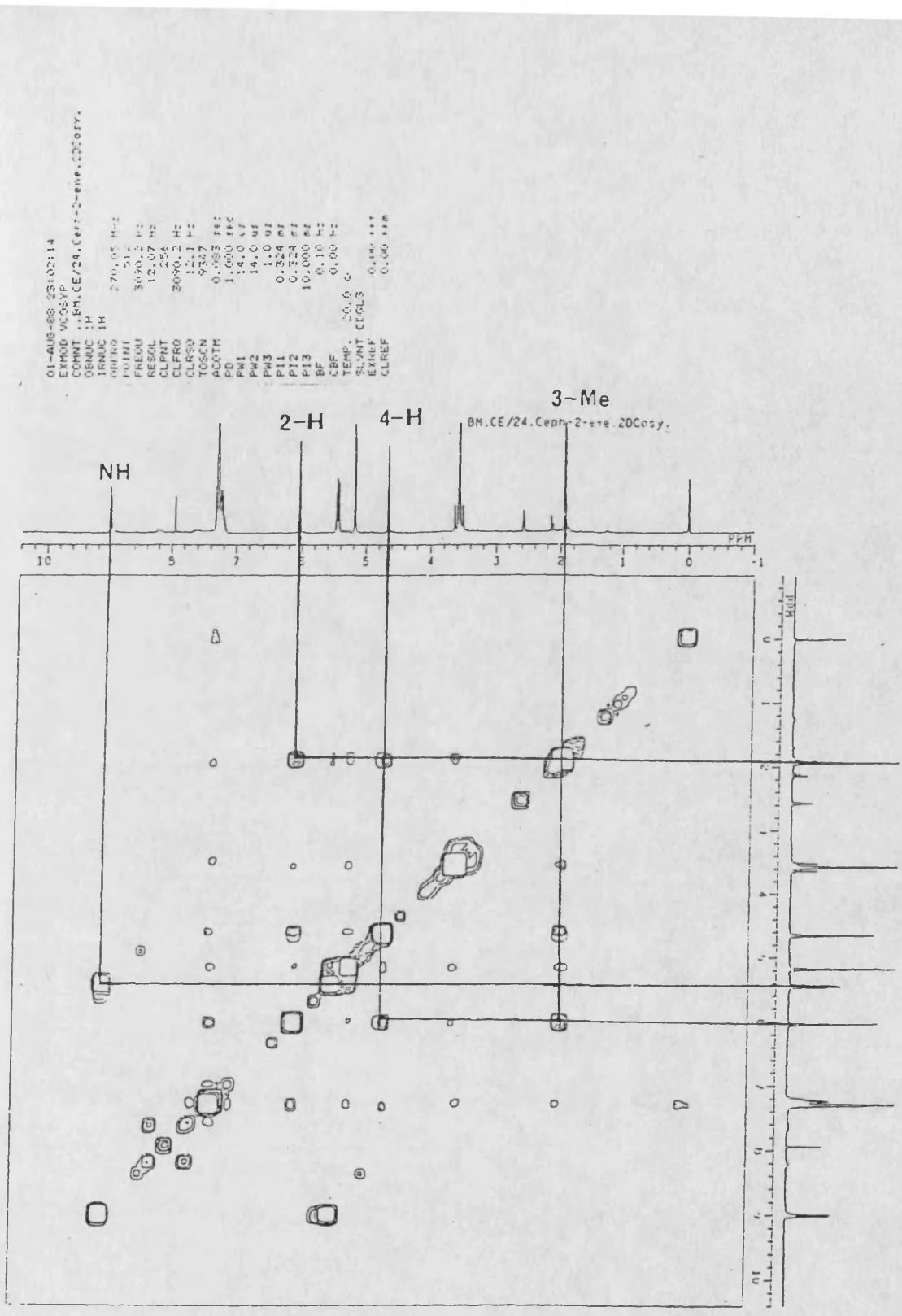


Fig 3.24 : 2D-^{1}H/^{1}H correlation spectrum of ceph-2-ene 3.8 in $CDCl_3$ + DMSO-d_6. (Experimental conditions as in section 3.3.1 and p.105).

2. Off-resonance proton decoupling of 3.8 in ($CDCl_3$+DMSO-d_6)

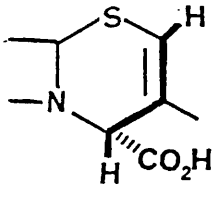
In the normal ^1H NMR spectrum (Fig. 3.21, p.100) the signal at 6.1 ppm due to 2-H appeared as narrow triplet (i.e. small couplings evident), that due to 4-H (at 4.7 ppm) as broad singlet, and the 3-Me signal at ~ 2.0 ppm as broad singlet. Only low power was used since the couplings between 2-H, 3-Me, and 4-H are long-range and therefore small (Fig. 3.25a-c, p.108).

a) Selective irradiation at the frequency of 2-H (at 6.1 ppm) : the signals at 4.7 ppm and 1.94 ppm appeared more sharper, due to decoupling the 2-H from the 4-H and 3-Me protons (Fig. 3.25a).

b) Selective irradiation at the frequency of 4-H (at 4.7 ppm) : the signal at 6.1 ppm changed to a narrow doublet with similar separation as that of 3-Me at 1.94 ppm, which indicates the long-range coupling of 2-H with 3-Me (Fig. 3.25b).

c) Selective irradiation at the frequency of 3-Me (at 1.94 ppm) : the signals at 6.1 ppm and 4.7 ppm appeared as narrow doublets with almost equal J values, i.e., showing long-range coupling between 2-H and 4-H (Fig. 3.25c).

The above two experiments (COSY and proton decoupling) provide clear evidence for long-range couplings (J~ 1 to 3 Hz) between the 2-H, 3-Me, and 4-H of the ceph-2-ene 3.8, and according to Sternhell[113], protons separated by four bonds (e.g. the case of 2-H and 4-H) showing coupling constant magnitudes of 1 to 3 Hz usually have a planar zig-zag arrangement, or a W configuration :



This arrangement is only possible when 4-H has a β-configuration. Hence, 4-CO_2H of the ceph-2-ene derivative 3.8 must have the α-configuration, i.e., the same as that of natural penicillin antibiotics (Fig. 3.23, p.104). Therefore, this physical study confirms Van

Heyningen's chemical findings about the configuration of ceph-2-enes at C-4.

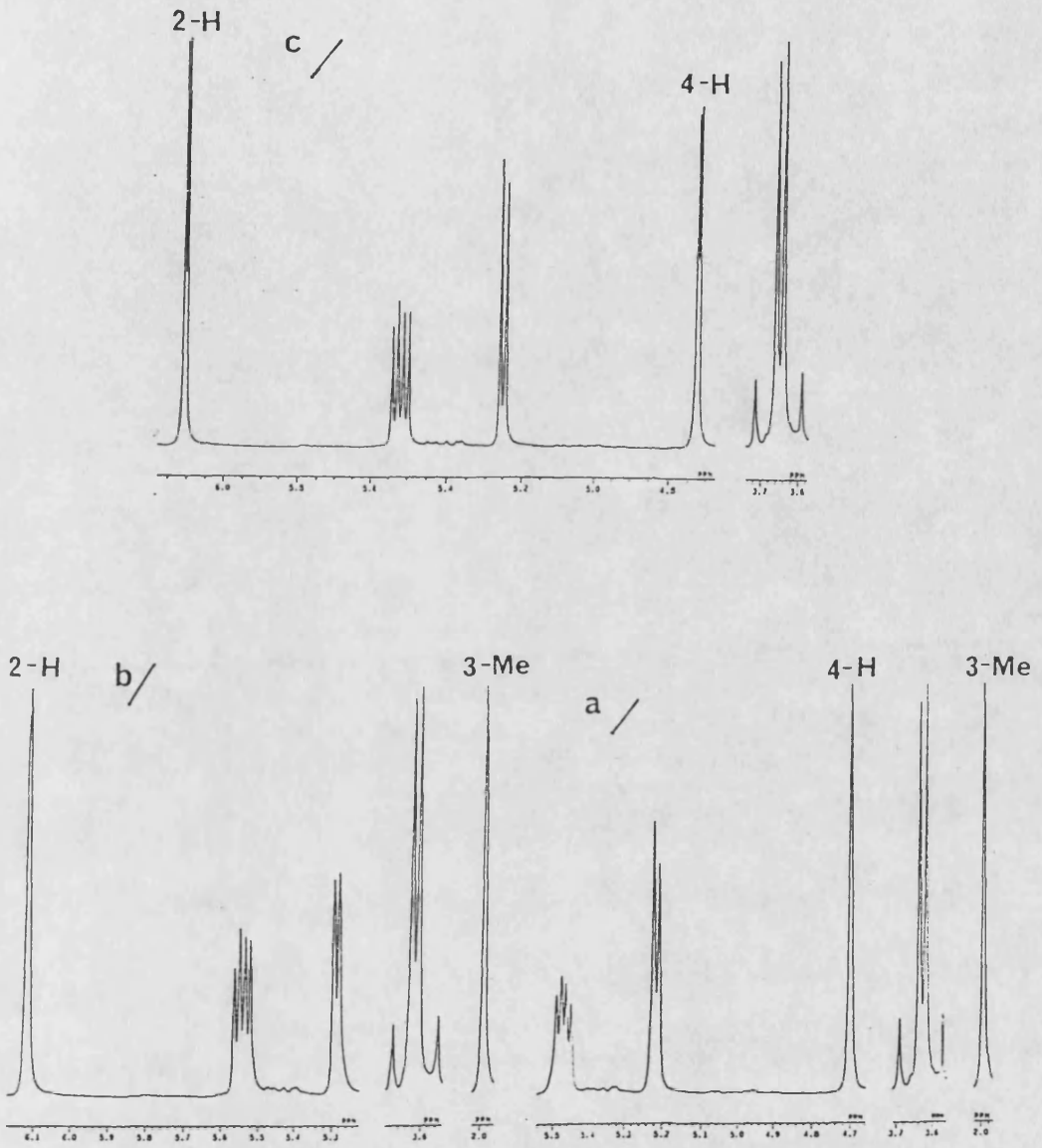


Fig. 3.25 : Off-resonance proton decoupling spectra of 38 in $CDCL_3$ + DMSO-d_6 illustrating effect of

a) selective irradiation at the frequency of 2-H (at 6.1 ppm),

b) selective irradiation at the frequency of 4-H (at 4.7 ppm), and

c) selective irradiation at the frequency of 3-Me (at 1.94 ppm) on long-range coupling between 2-H, 4-H and 3-Me.

CHAPTER FOUR

Stability and Degradation Investigations of β-lactam Antibiotics

4.1 Introduction

Numerous studies of the degradation of β-lactam antibiotics have appeared in the literature. Access to details can be obtained from the review articles referenced in the section. Many β-lactam antibiotics are labile in aqueous solutions. Hydrolytic fission of the β-lactam ring produces an inactive drug.

4.1.1 Penicillins

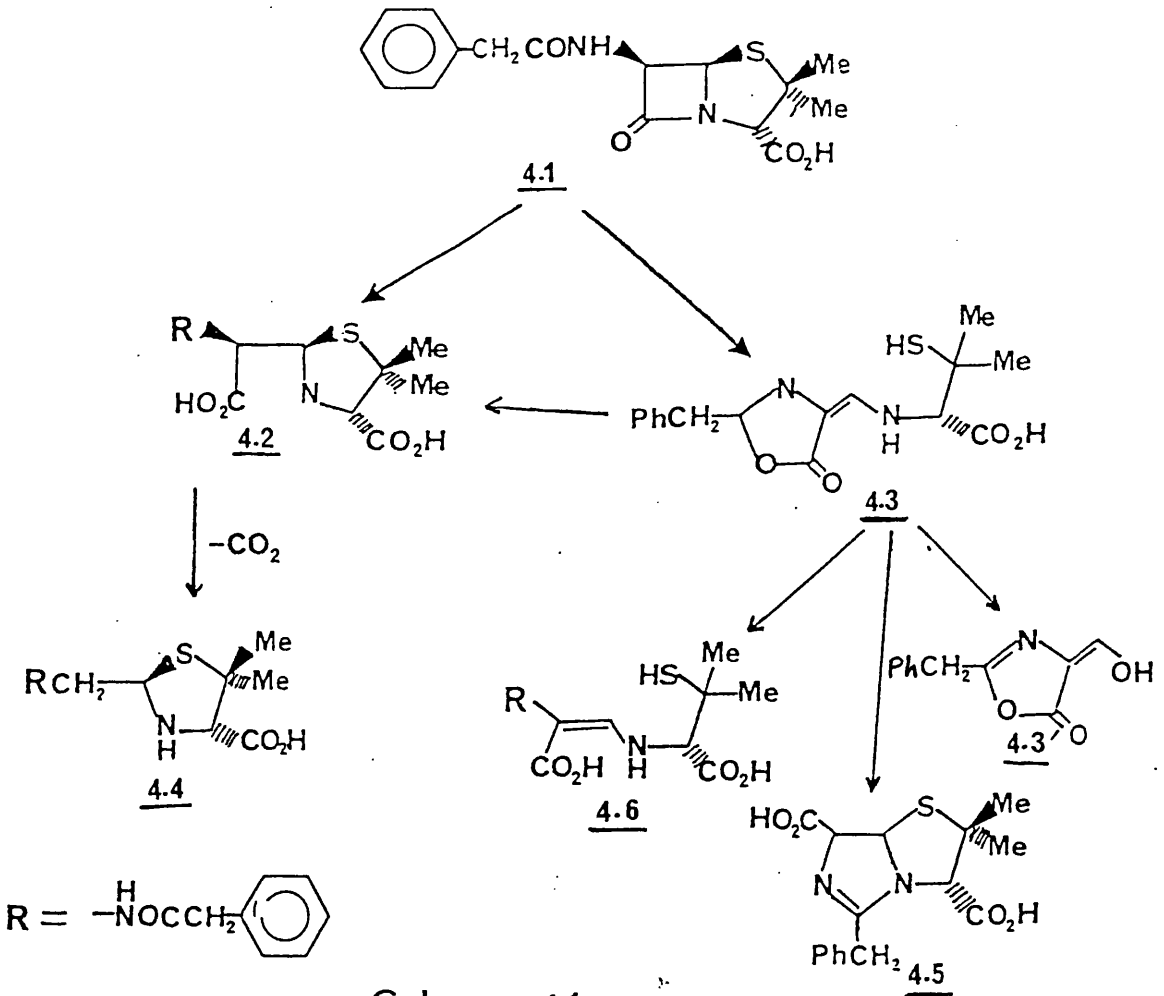
An extensive degradative study of benzylpenicillin is described by Clarke et al.[61]. In aqueous solution. the penicillins are not only degraded by acid and base, but also by metal ions, penicillinase enzymes, oxidizing agents, alcohols, amines, and a wide range of other nucleophiles and electrophiles[27,61].

The penicillins differ only with respect to the acyl side chain on C-6; the remainder of the molecule is the same in all penicillins except pro-drug formulations. In what follows only the degradation of benzylpenicillin will be considered.

4.1.1.1 Acid-base hydrolysis of benzylpenicillin

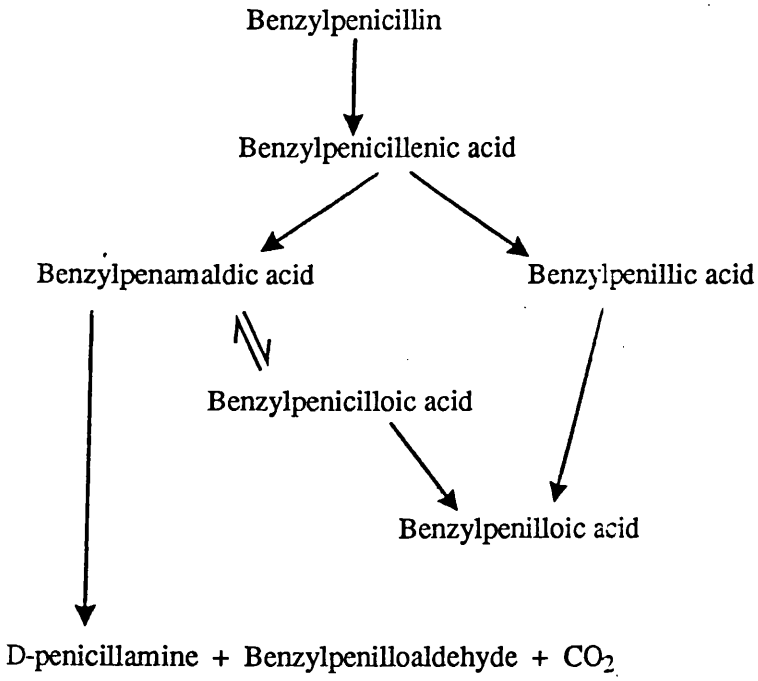
The degradation has been found to be first-order with respect to benzylpenicillin in acidic, neutral and basic aqueous solutions[50,114]. The pH-rate profile showed that penicillin solution was moderately stable at pH 5 to 6, but labile towards acid and alkali[50,115]. The overall rate of degradation of benzylpenicillin in aqueous solutions is proportional to temperature and found to obey the Arrhenius expression[50] (i.e. Temperature dependence-activation reaction or energy, expressed by the equation $\frac{[dlnK]}{[dt]} = \frac{E}{RT^2}$, where K is the reaction rate in terms of concentration, T=temperature, E=energy of activation).

The instability of benzylpenicillin 4.1 in acidic aqueous solution is well known[6], and its major degradation product, benzylpenillic acid 4.5, fully defined[6.1]. Other observed degradation products include, benzylpenicilloic acid 4.2, formed when the β-lactam ring opens and benzylpenicillenic acid 4.3, detected by its intense UV absorption at 322 nm[116,117]. It was later proposed[118] that benzylpenicilloic acid, and its product of decarboxylation in acidic solution (benzylpenilloic acid 4.4), are also important degradation products (see Scheme 4.1 below). Various studies indicate that benzylpenicillenic acid is highly unstable, and is rapidly hydrolyzed not only to benzylpenillic acid[150] but also to benzylpenamaldic acid 4.6[49,117], benzylpenicilloic acids[151] and 2-benzyl-4-hydroxymethyl-ene- oxazol-5(4H)-one 4.3'[11].



Scheme 4.1

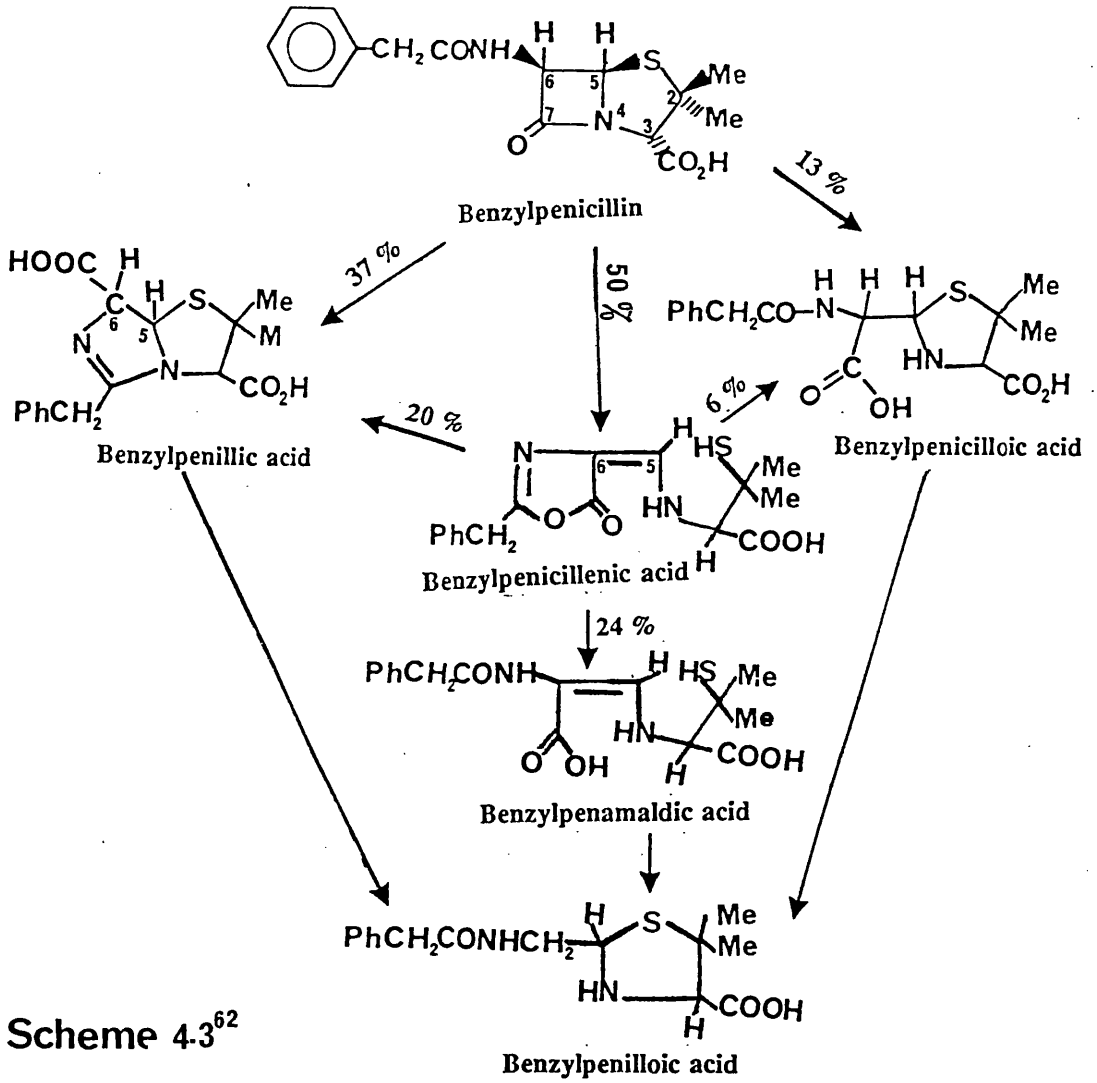
Several workers have monitored degradations of benzylpenicillin by high-performance liquid chromatography (HPLC). Blaha and co-workers[37] employed ion-exchange chromatography to study benzylpenicillin degradation at pH 2.7 and 37°C. In this study, benzylpenicilloic acid was not detected and this fact was attributed to its existence in equilibrium with benzylpenamaldic acid and its rapid decarboxylation to produce benzylpenilloic acid. A degradation Scheme was proposed (Scheme 4.2 below) based upon the order of appearance of the degradation products:



Scheme 4.2

Vanderhaeghe and co-workers[38] utilised cation-exchange chromatography to separate a comprehensive series of decomposition products derived from benzylpenicillin. Kessler et al.[39] examined the influence of ion-pair reagents on the degradation of benzylpenicillin at pH 2.5 and 37°C.

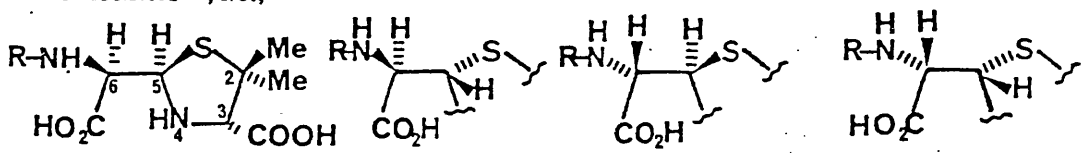
Degalaen and others[62] have monitored the degradation of benzylpenicillin at pH 2.5 and 37°C by ^{1}H NMR spectroscopy. Signal assignments were made by comparisons with spectra of standards recorded under the same conditions. Signals due to penillic, penicilloic and penamaldic acids appeared in the early stages of degradation. From the second day, lines due to penilloic acid steadily increased with concomitant fall in the intensities of signals due to the initially formed acids. After 30 days the spectrum was essentially that of penilloic acid (Scheme 4.3).



Scheme 4.3[62]

From the above ^{1}H NMR study it was shown that benzylpenicillenic acid is not a major intermediate, as proposed by Blaha[37], in the formation of benzylpenillic in

solutions[45,50,119–121]. In general, all the observations reported by these workers may be summarised as follows. Results indicated that the mechanism of alkali-catalysed degradation of benzylpenicillin is of nucleophilic attack of OH^- on the β-lactam ring[119]. It was found that the alkali-inactivated product was benzylpenicilloic acid 4.2 which may exist as a mixture of isomers[45], i.e.,

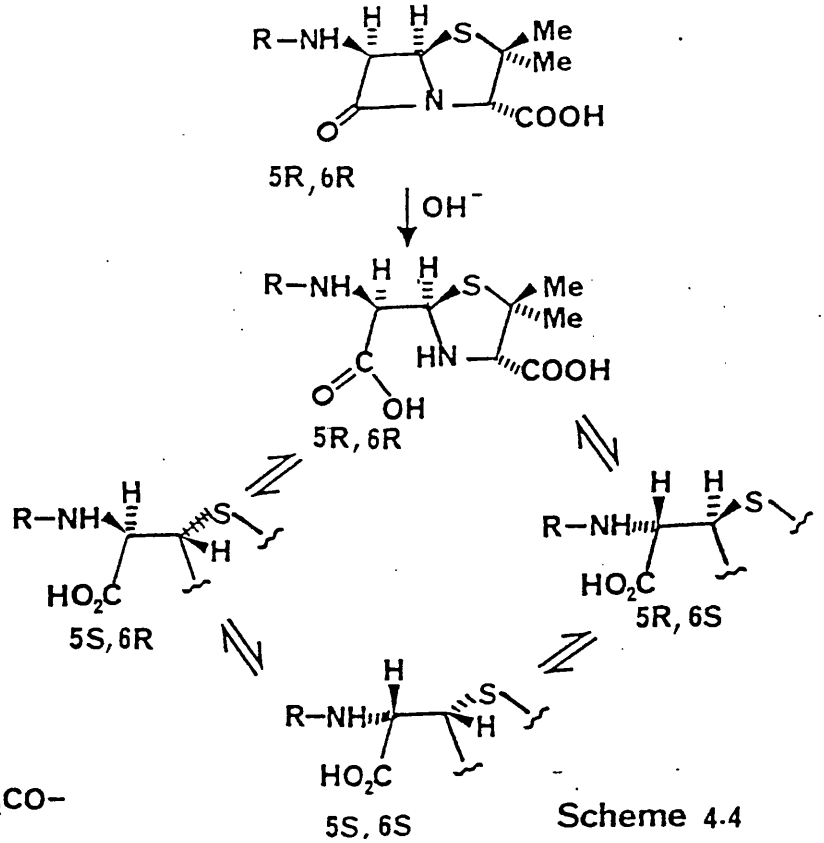


4.2A (3S,5R,6R) 4.2B (5S,6R) 4.2C (5R,6S) 4.2D (5S,6S)

R= $PhCH_2CO-$

Ressler et al.(1985)[122] reported an HPLC method for the separation of the four stereoisomers of 'natural' benzylpenicilloic acid. It was concluded that epimerization at C-5 was the major isomerization process but it is apparent from the reported data that epimerization at C-6 is also possible.

Scheme 4.4 summarises the alkaline degradation of benzylpenicillin involving the cleavage of the β-lactam ring to produce benzylpenicilloic acid and its epimers.

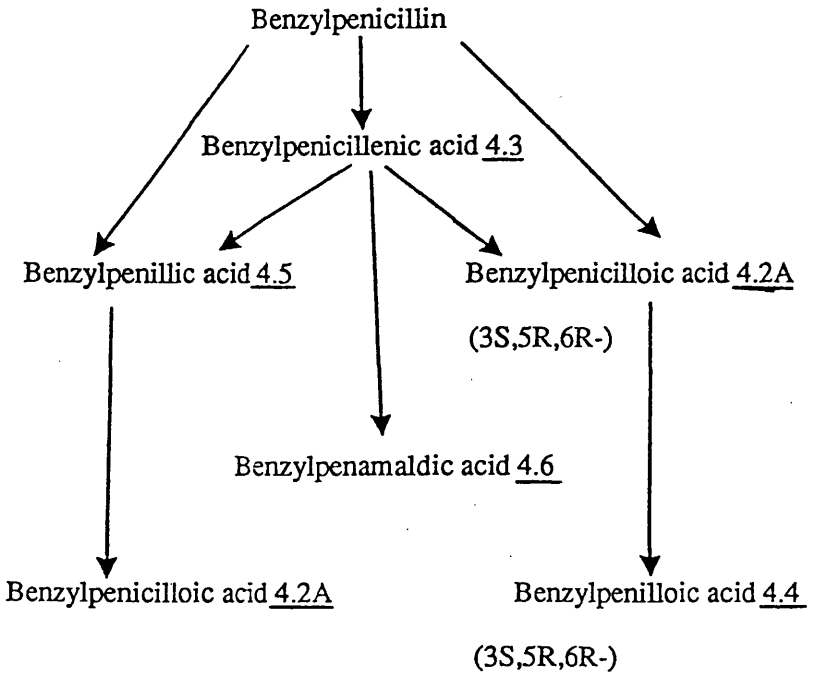


R= $PhCH_2CO-$

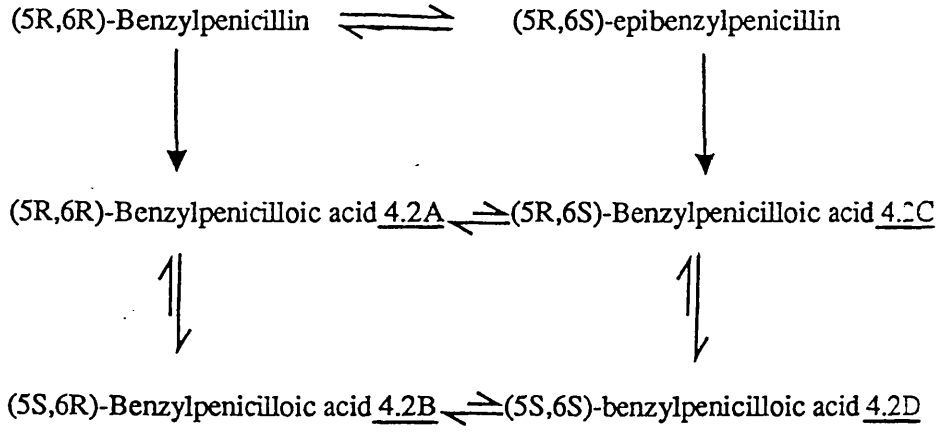
Scheme 4.4

More recently Lipczynski (1988)[85] reported a combined NMR-HPLC investigation of the degradation of benzylpenicillin in acidic and basic aqueous solutions. The author aimed to clarify the uncertainty about the details of the degradation of benzylpenicillin and the inter-relationships between the various degradation products, and to provide a kinetic study of the entire degradation process and reaction mechanisms consistent with experimental observations. The study reports employment of quantitative HPLC systems and NMR methods to optimise the separation and identification of benzylpenicillin degradation products. The degradation pathways proposed by the author are summarised in Schemes 4.5 and 4.6 below.

Benzylpenicillin degraded in acidic solution to benzylpenillic acid 4.5, (3S,5R,6R)-benzylpenicilloic acid 4.2A and benzylpenicillenic acid 4.3. The dominant breakdown product of 4.5 was found to be 4.2A, which is good evidence for 4.5 possessing (3S,5R,6R) stereochemistry, this not having been reported previously. Benzylpenicilloic acid in acidic solution was found to exist in complex equilibria with its C-5 and C-6 stereoisomers, benzylpenillic acid 4.5, and benzylpenamaldic acid 4.6; the dominant degradation pathways were epimerisation at C-5 and stereoselective decarboxylation to benzylpenilloic acid 4.4. Benzylpenicillenic acid 4.3 was found to be highly unstable in acid solution. The dominant breakdown products were benzylpenillic acid 4.5, the four possible (3S)-benzylpenicilloic acid stereoisomers 4.2 and benzylpenamaldic acid 4.6 (see Scheme 4.5, p.115). In basic solution benzylpenicillin was hydrolysed to (3S,5R,6R)-benzylpenicilloic acid 4.2A and also epimerised at C-6 to 6-epibenzylpenicillin which in turn was hydrolysed to (3S,5R,6S)-benzylpenicilloic acid 4.2C (Scheme 4.6, p.115). The author[85] also reported the preparation of a number of the above-mentioned degradation products, which were used to investigate the kinetics and mechanisms of the individual steps in the degradation sequence by HPLC, and, together with UV and ^{1}H NMR spectroscopy, used to monitor the breakdown of benzylpenicillin and its degradation products in aqueous solution.



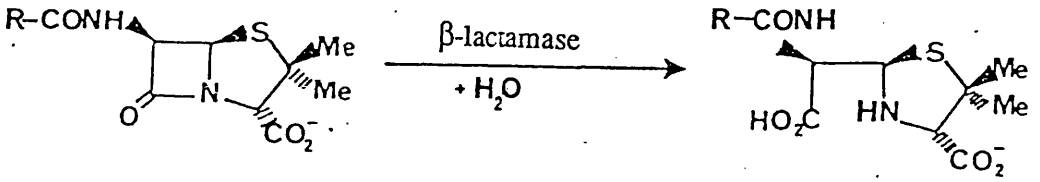
Scheme 4.5 : Degradation of benzylpenicillin in acidic solution[85]



Scheme 4.6 : Degradation of Benzylpenicillin in alkaline solution[85]

4.1.1.2 Enzymatic hydrolysis of benzylpenicillin

Benzylpenicillin appears to be susceptible to all known β-lactamases, being converted to inactive penicilloic acid. These enzymes hydrolyse the β-lactam ring[45].



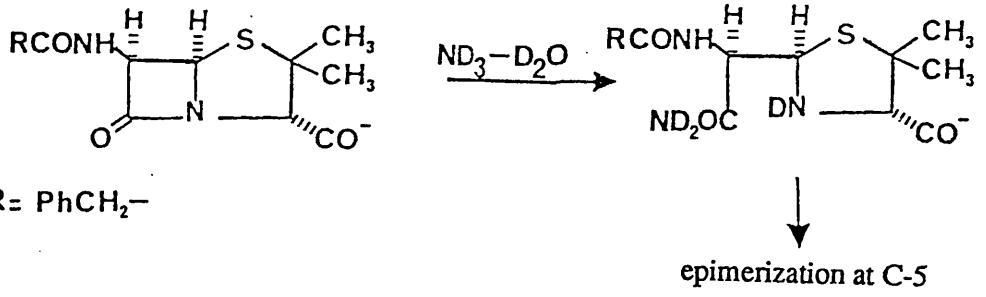
penicillin penicilloic acid

R= $PhCH_2$–

4.1.1.3 Aminolysis of benzylpenicillin

This reaction is of interest because the principal antigenic determinant of penicillin allergy is the penicilloyl group bound by an amide linkage to amino groups on proteins[123a,b]. This amide exchange reaction occurs readily and the susceptibility of penicillin to attack by nucleophiles has been attributed to strain in the β-lactam ring and the nonplanarity of the system which inhibits the usual amide resonance[61].

Hamilton-Miller and co-workers[44a,b] followed the aminolysis (ND_3 in D_2O) of benzylpenicillin by UV absorption and ^1H NMR spectroscopy. The changes in the absorption spectra (λ_{max}230 nm) and those of the ^1H NMR spectrum (upfield shift in the β-lactam proton signals and the increased non-equivalence of the gem-dimethyl groups), indicated the cleavage of the β-lactam ring, with rapid epimerization at C-5.

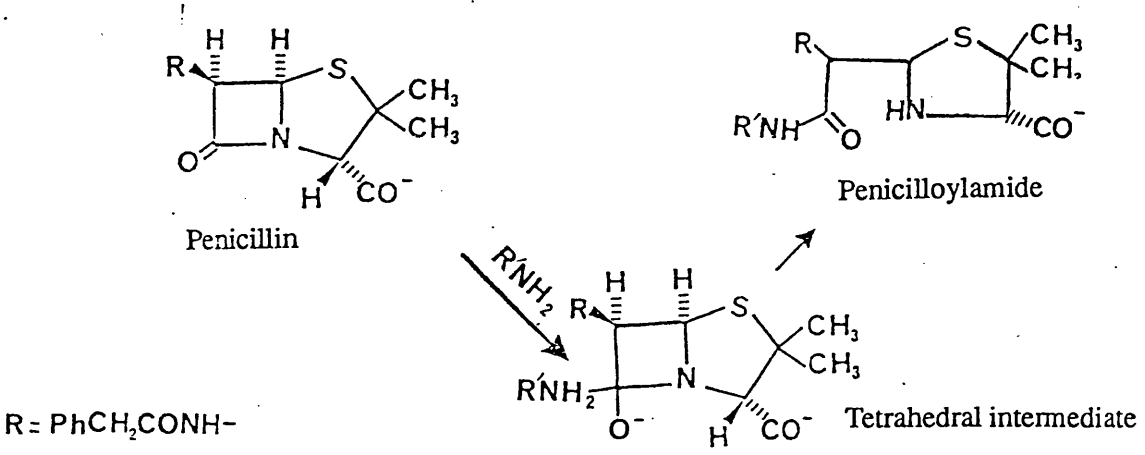


R= $PhCH_2$–

epimerization at C-5

No evidence was given for the epimerization at C-5.

Page and associates[124a,b] suggested that the aminolysis of benzylpenicillin proceeds through a tetrahedral addition intermediate, i.e., a two-step reaction.



The authors reported kinetic evidence of the tetrahedral intermediate involving a change in the rate-limiting step of the base-catalysed aminolysis reaction.

4.1.1.4 Hydrazinolysis of benzylpenicillin

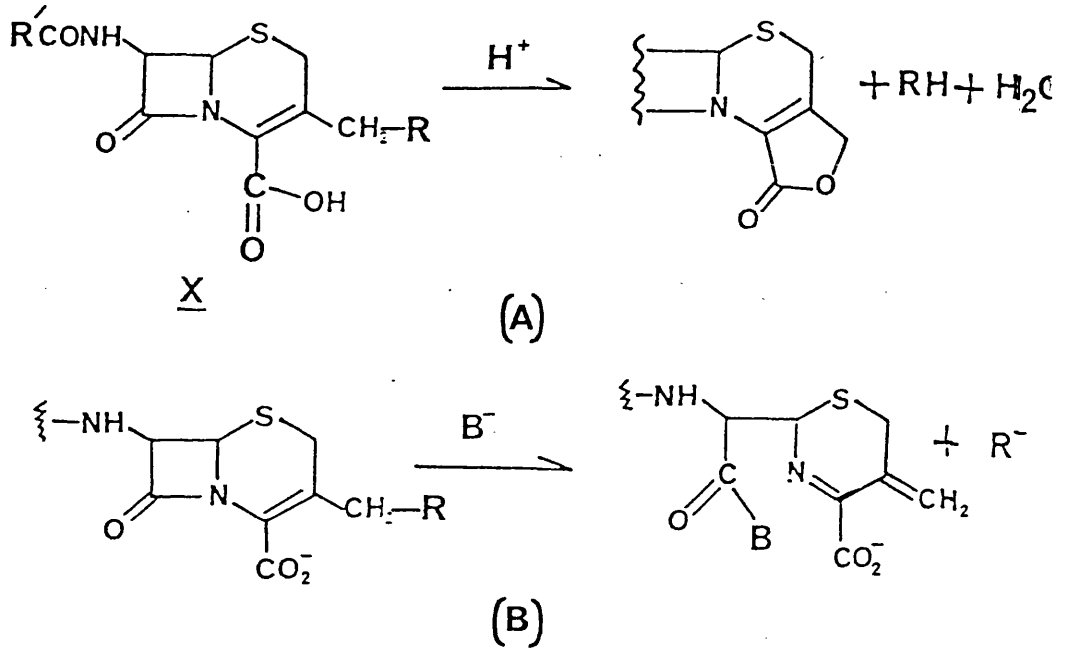
Morris and Page (1980)[124a,b] have shown that hydrazine is a very effective nucleophile towards the β-lactam carbonyl group. In their hydrazinolysis experiments, they used 25 µl of aqueous benzylpenicillin Na (0.1 M), added to 2.5 ml of 0.005 - 0.2 M hydrazine in presence of increasing concentrations of buffer solutions prepared from various salts, pre-incubated at 30°C, with thorough mixing. The disappearance of penicillin was followed spectrophotometrically at 235 nm. The authors suggested that the cleavage of the lactam ring proceeds through a similar way as that of aminolysis (section 4.1.1.3). They did not attempt to identify the degradation products to support their proposed mechanisms of hydrazinolysis.

4.1.2 Cephalosporin degradations

4.1.2.1 Simple degradation studies

Several studies of the degradation of cephalosporin antibiotics have been made which involve simple monitoring of decrease in the concentration of the antibiotic with time by use of HPLC and other analytical techniques. No product identifications were sought in these

studies. Cephalosporins of the type X, in general, easily undergo acid and base hydrolysis[4] (Scheme 4.7) :



R = good leaving group

R′= benzyl and related groups

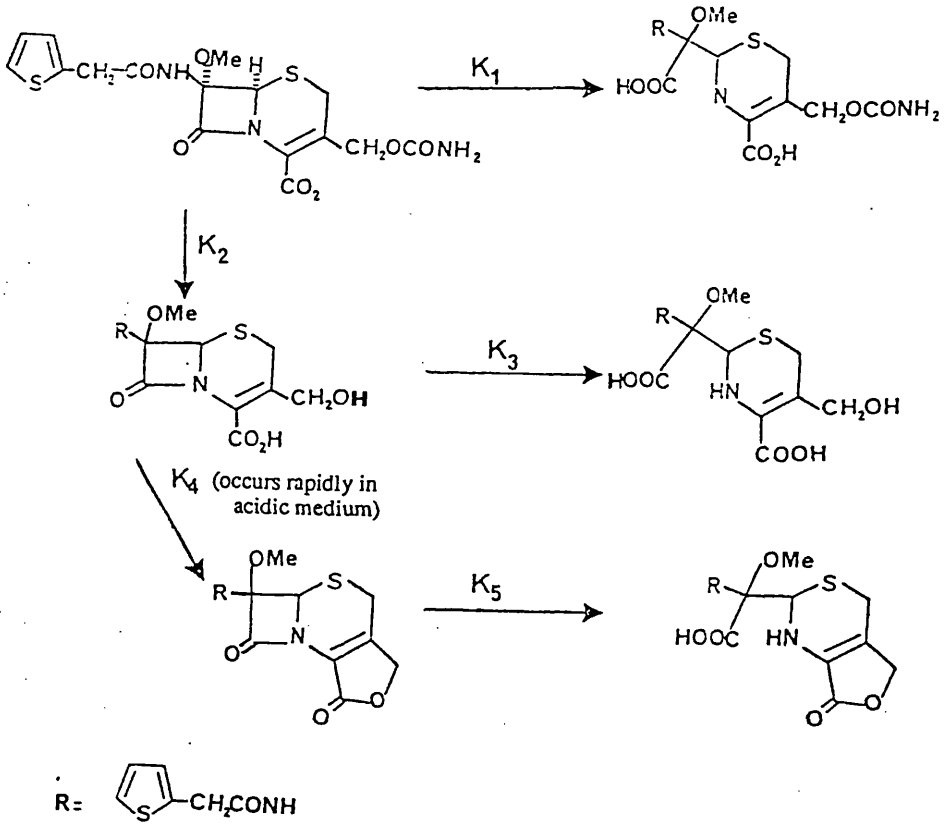
Scheme 4.7

Acid hydrolysis results in the loss of the R substituent (a leaving group) at the 3-position and formation of an α, β-unsaturated lactone. Base hydrolysis opens the β-lactam moiety and (simultaneously?) eliminates the R group at the 3'-position, with double bond migration. Enzymatic deactivation of cephalosporins is believed to follow a reaction path similar to that of base hydrolysis, again with the loss of R group[4]. In general, the degradation of cephalosporins follow apparent first-order kinetics[36,47].

Jones et al. (1968)[125] and Hall(1973)[69] reported the use of a polarographic technique to follow acid and base hydrolysis and β-lactamase degradation of some cephalosporins, i.e., cephalosporin C, cephalothin and cephaloridine. The degradation mechanism followed those pathways of Scheme 4.7. The polarographic method's main limitations are the requirements of reducible compounds, and the fact that it is only applicable to cephalosporins containig a

leaving group at the 3-position.

Das Gupta[40] employed UV and HPLC techniques to investigate the kinetics of the hydrolysis of cefoxitin in aqueous solutions at 24°C and different pH value buffers, buffer concentrations and ionic strengths. The pH-rate profile showed that cefoxitin solution was moderately stable at pH 4-7, the major reaction being the hydrolysis of the β-lactam ring by water, but very labile towards acid and alkali. The study reports the use of two different columns and mobile phases to confirm the reliability of the HPLC method over that of the UV spectrophotometry. The proposed degradation pathways of cefoxitin are shown in Scheme 4.8 below:

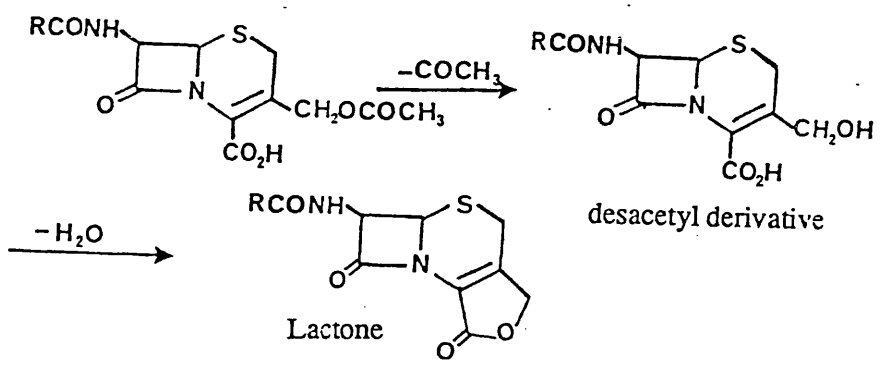


Scheme 4.8

Increases in buffer concentration or ionic strength had very little effect in the decomposition rate at the various pH values.

4.1.2.2 Degradation of 3-acetoxymethyl derivatives to lactones

These derivatives may lose their acetyl function to give corresponding desacetyl derivatives which subsequently eliminate water to form the fairly stable lactones (Scheme 4.9). This route is well documented in the literature.



Scheme 4.9

The rapid lactonization of the desacetyl derivative to the lactone occurs only at relatively low pH.

The chemical and/or enzymatic conversion of cephalosporin C 4.7a[47] and of several clinically useful 3-acetoxymethyl derivatives, cephalothin 4.7b[36], cephaloglycin 4.7c[125,127] and cefotaxime 4.7d[75,128], into the corresponding desacetyl analogues 4.8 and lactones 4.9 has been described (Fig. 4.1, p.121). Two pathways have been proposed[129] by which the nonenzymatic hydrolysis of these cephalosporins, or of any ester, may proceed. The more common path involves acyl-oxygen bond cleavage, but alkyl-oxygen bond cleavage may occur if the intermediate carbonium ion is particularly stable[129].

Yamana and Tsuji[36] followed the aqueous acidic degradation of some 3-acetoxymethyl cephalosporin derivatives by HPLC and other techniques. The acetyl functions of the derivatives were found to hydrolyse eight times faster than their β-lactam moieties to yield the corresponding desacetyl intermediates, which were rapidly converted to the lactones. The authors proposed Scheme 4.10 (p.121) for the entire pH range; k_1, k_3, and k_5 correspond to the rates of β-lactam cleavage in the nucleus. In neutral and basic pH regions, the k_4

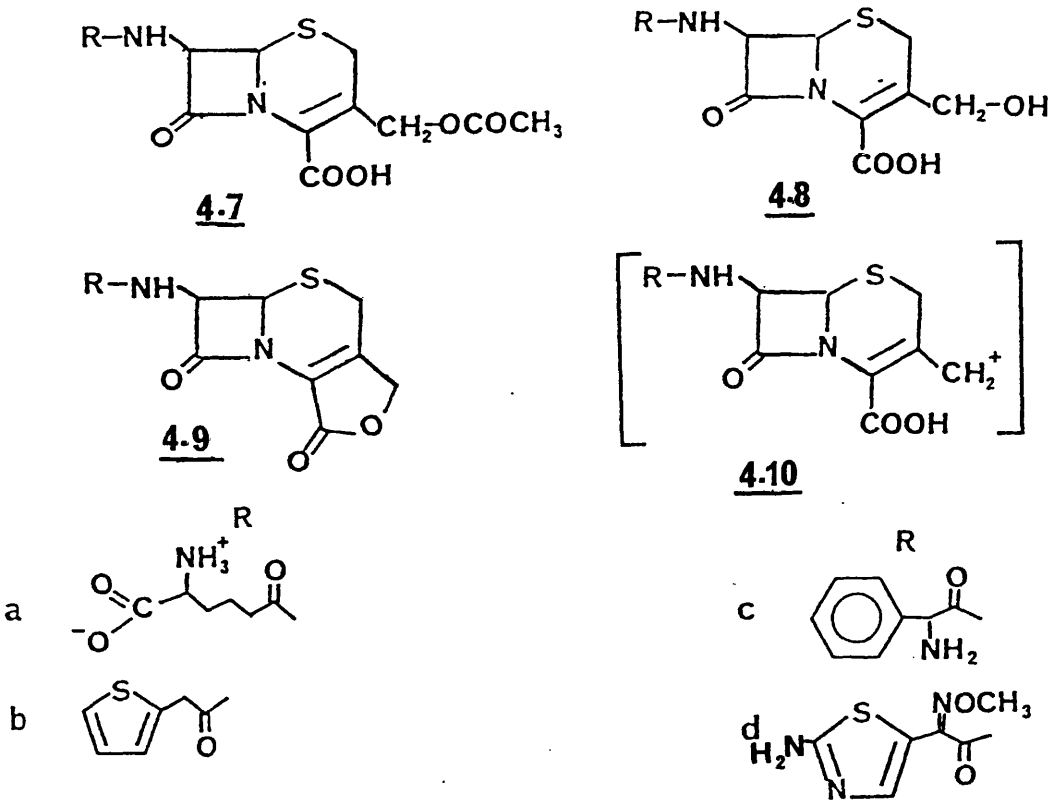
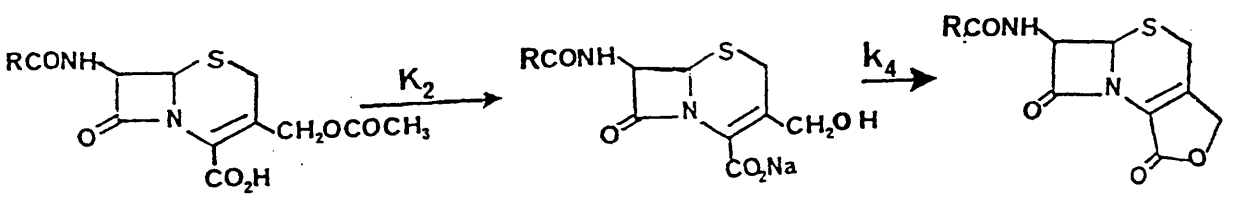


Fig. 4.1 : Chemical structures of some clinically useful 3-acetoxymethyl cephalosporin derivatives and their corresponding desacetylanalogues and lactones.



Scheme 4.10

reaction process is negligible, but became significant below pH 4.0. For example, for cephalothin the value of k_4, at pH 2 and 35°C, was 0.708 hr^{-1}. No mechanism was suggested for the loss of the acetyl group to give the corresponding desacetyl derivative.

Mangia and co-workers[71] reported a quantitative HPLC method for the determination of cephacetrile in bulk drugs and pharmaceutical formulations. They suggested the superiority of their method over the official microbiological and colorimetric method, in being more selective, rapid, accurate and free of interference by acetyl hydrolysis products. Furthermore, the desacetyl and lactone derivatives, arising from cephacetrile during storage, can be detected accurately upto 0.1%.

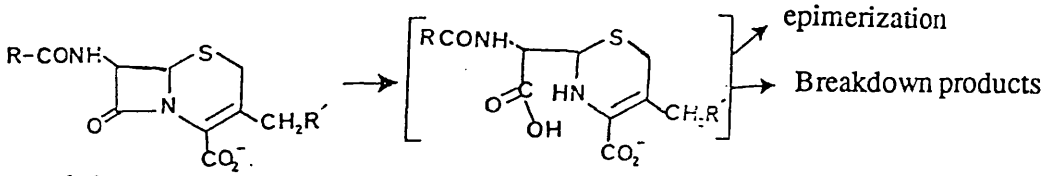
The mechanisms for the aqueous formation of a desacetyl derivative from a 3-acetoxymethyl cephalosporin 4.7 have received much attention. Cocker et al.[130] and Taylor[131] suggested the existence of a resonance-stabilized allylic cation 4.10 (Fig. 4.1, p.121) resulting from alkyl-oxygen bond cleavage, to explain sulphur and nitrogen nucleophilic displacement reactions at the allylic carbon in aqueous solution.

Hatfield et al.[132] suggested that 4.10 is also an intermediate in similar nonaqueous nucleophilic displacements. None of these workers, however, succeeded in isolating 4.10 or its nucleophilic reaction products. The only observed reaction was destruction of the β-lactam ring. Hence, Hatfield and co-workers proposed an acyl-oxygen bond cleavage pathway rather than an alkyl-oxygen bond cleavage or allylic cation 4.10, for the formation of the desacetyl derivative 4.8 and its subsequent cyclization product, lactone 4.9, under aqueous conditions (Fig. 4.1).

More recently, Indelicato et al.[72] reexamined the aqueous solution chemistry of the C-3'-acetoxy group of cephalothin sodium 4.7b with isotopically labeled $H_2{}^{18}O$ and [2-^{13}C] acetate anion. They suggested, from the ^{18}O incorporation studies, that the hydrolysis (at pH 4.7) of 4.7b to the desacetyl derivative of cephalothin 4.8b proceeds via

two pathways : ~ 55-63% of the reaction occurs via alkyl-oxygen cleavage (C_3-CH_2--OR) or allylic cation 4.10b (Fig.4.1, p.121) and the remaining via acyl-oxygen cleavage (C_3-CH_2O--R). They observed that the alkyl-oxygen cleavage pathway is a reversible reaction.

4.1.2.3 β-Lactam ring opening



cephalosporin unstable intermediate

Scheme 4.11

This route (Scheme 4.11) is anticipated from studies of penicillin derivatives and should lead to analogues of penicilloic 4.2 and penilloic 4.4 acids together with their products of epimerization. The situation with cephalosporins is more complicated owing to the presence of another substituent on the nucleus at position 3. Unlike 4.2 and 4.4, the cephalosporin analogues are usually very unstable and can not be isolated because they undergo rapid decomposition[133]. To date cephalosporin analogues of 4.2 and 4.4 have only been isolated in the case of cefixime (Namiki et al., 1987)[70]; there is no ^{1}H NMR evidence for their existence. Two other exceptions[134] known to this are, one being cephalosporins where the 3-substituent is linked within a γ-lactone[4] (Fig. 4.2a); the other comprises compounds where the 3-substituent is the highly conjugated system of 2:4- dinitrostyrene[135], (Fig. 4.2b) :

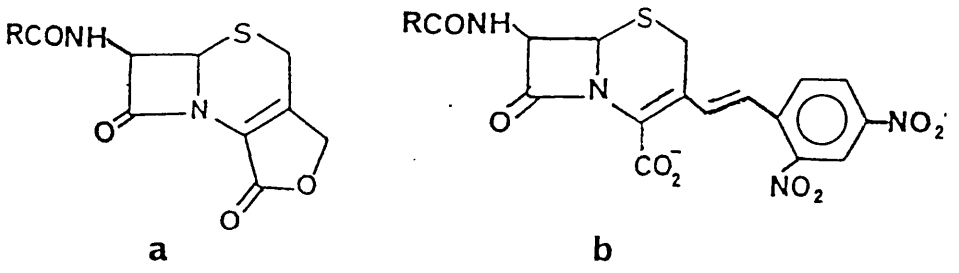
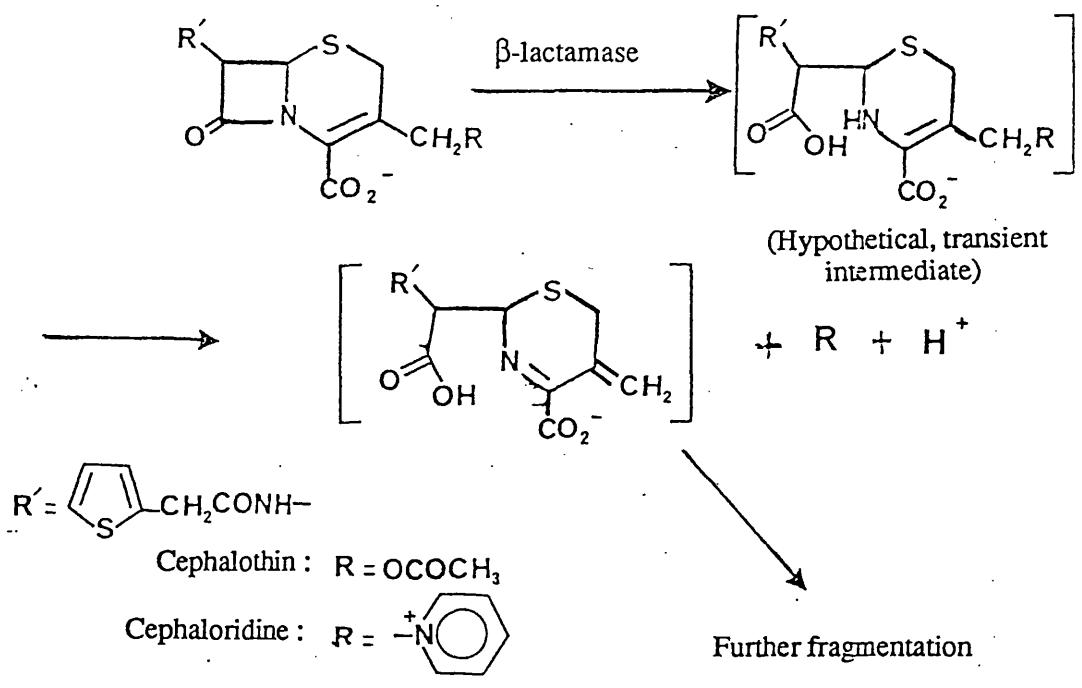


Fig. 4.2 : a) a cephalosporin lactone; b) 3-(2:4-dinitrostyrene) cephalosporin derivative.

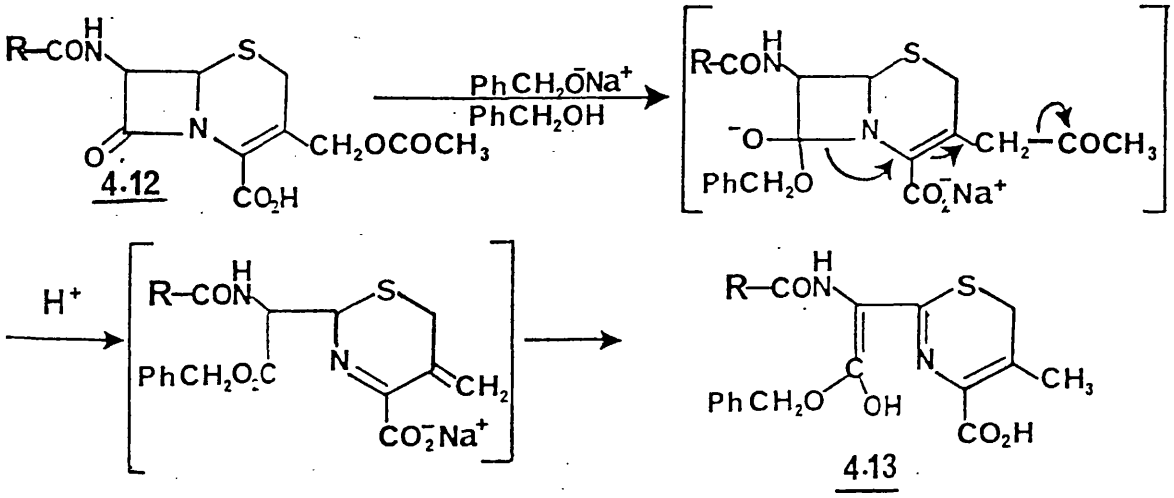
Sabath and co-workers (1965)[41] reported that β-lactam cleavage of cephalosporin C, cephalothin and cephaloridine with a β-lactamase was accompanied by the appearance of two equivalents of acid per mole of antibiotic, in case of the former two cephalosporins; one equivalent was attributed to the β-lactam hydrolysis while the other resulted from the liberation of acetate ion. Cephaloridine liberated, in addition to one equivalent of acid, one equivalent of base derived from pyridine (attached to C-3'). UV spectra of product solutions showed that the original 260 nm absorption disappeared as a 230 nm maximum appeared which, in turn, disappeared with time. The exact structures of the degradation products were not determined (see Scheme 4.12 below).



Scheme 4.12

Compounds such as cephazolin and cephamandole probably lose their 3-substituent, but this reaction may not proceed as rapidly as the decomposition of the β-lactam ring, and the nature of the decomposition products is not yet firmly established.

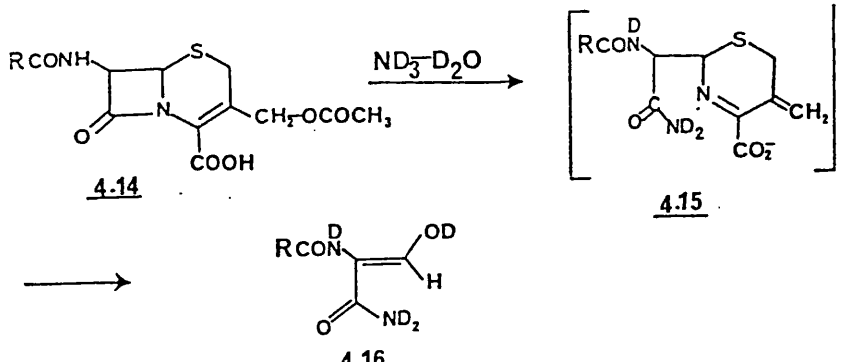
Eggers and associates (1965)[136] studied the reaction of cephaloram 4.12 with sodium benzyloxide in benzyl alcohol. The product 4.13, which has lost the acetoxy function, was suggested to arise by Scheme 4.13 illustrated below. The structure of compound 4.13 was verified by independent synthesis.



R = $-CH_2Ph$

Scheme 4.13

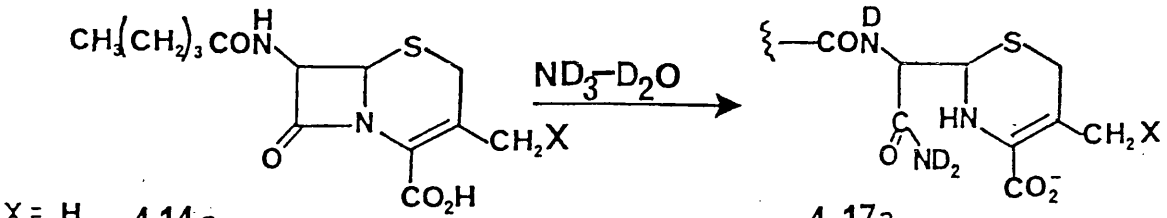
Studies by Hamilton-Miller et al. (1970)[44a,b] on the aminolysis and enzymatic hydrolysis of cephalosporins have shed considerable light on the complex breakdown pattern of the cephalosporins. The degradation of 7-n-butyramidocephalosporanic acid 4.14 with ND_3-D_2O was monitored by NMR and UV spectra. Analysis of the data and comparison with model compounds led to the conclusion that the exo-methylene compound 4.15 was formed as a semistable intermediate. Compound 4.15 (λ_{max} 230 nm) underwent further degradation to a new compound (λ_{max} 270 nm) which was assigned structure 4.16, (Scheme 4.14).



R = $-(CH_2)_3CH_3$

Scheme 4.14

However, aqueous aminolysis of the desacetoxy (a) and desacetyl (b) derivatives of 4.14, gave intermediate desacetoxy 4.17a and desacetyl 4.17b cephalosporoates ($\lambda_{max} \neq 230$ nm), (Scheme 4.15).



X = H 4.14a
X = OH 4.14b

4.17a
4.17b

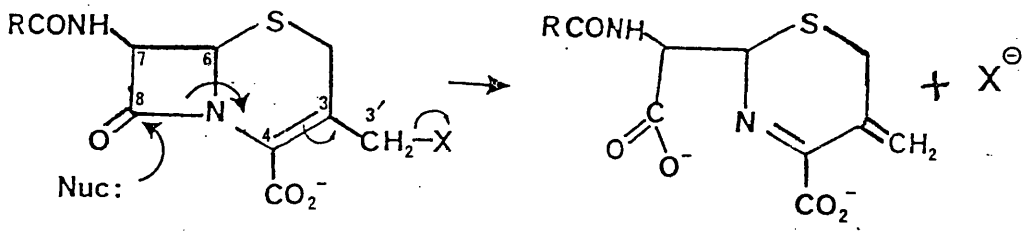
Scheme 4.15

β-lactamase treatment of desacetylcephalosporins, gave intermediates with λ_{max} 230 nm, which is a different result from the aminolysis. The investigators speculated that enzymatic treatment could have converted the hydroxyl groups of the desacetyl compound into better leaving groups resulting in behaviour similar to that of cephalosporins.

4.1.2.4 Elimination of a leaving group from the 3-position of a cephalosporin (Formation of 3-methylene derivatives)

It is well known[42,137–139] that the antibacterial effectiveness of cephalosporin antibiotics is strongly influenced by substituents at the 3-position. Furthermore, these substituents may have additional influence through their leaving group ability since it is known[41,44a,b,133] that nucleophilic β-lactam ring cleavage of cephalosporins that have a good leaving group in the 3-position, as many clinically important cephalosporins in fact do possess, is accompanied by elimination of the leaving group. Degradation products of this kind involve cleavage of the β-lactam ring coupled to migration of the N-CO bond electrons into the dihydrothiazine ring, shift of the endocyclic 3-4 double bond to the 3-exocyclic position and expulsion of the terminal group X as an anion (Scheme 4.16, p.127). The

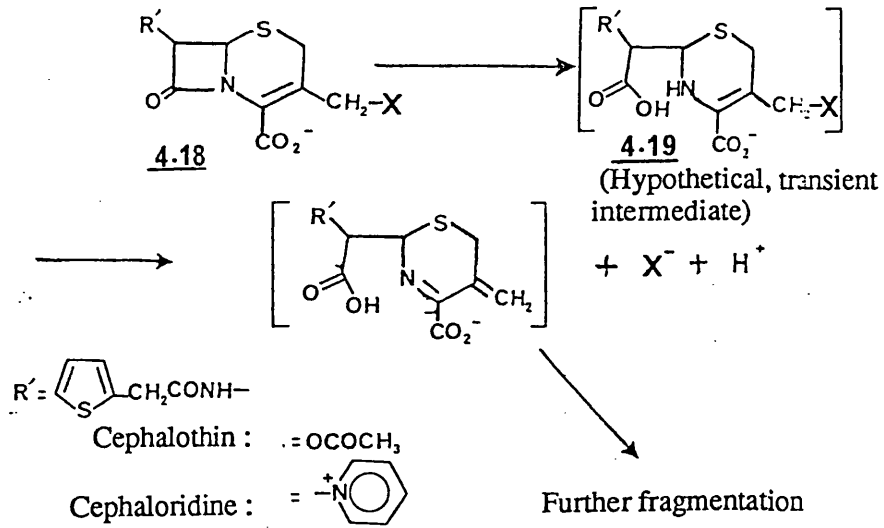
process requires X to be a group which forms a stable anion, i.e., has good "leavability" properties.



3-methylene cephem

Scheme 4.16

Until 1984, both experiment[140–142] and theory[42,57,143] have been interpreted in terms of β-lactam ring opening concerted with departure of the leaving group X in 4.18; i.e., no intermediate corresponding to 4.19, as shown in Scheme 4.17, has been observed in cases where X is a good leaving group such as acetoxy or pyridinium :



Scheme 4.17

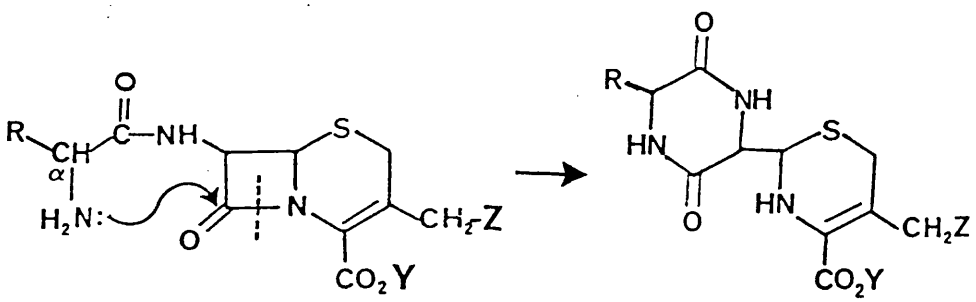
Faraci and Pratt (1984)[59] followed the reaction of a β- lactamase (TEM-2 type) with cephaloridine spectrophotometrically, at 260 nm. They reported a two-phase proton release reaction. They anticipated that one proton would be released during each step of Scheme

4.12 (p.124), as it is well-known that two protons are released on hydrolysis of cephaloridine[41]. They concluded that, the departure of a good leaving group need not in general be concerted with β-lactam ring opening. No product identifications were sought in this study.

Recently, intervention of the intermediate (with the 3'-leaving group) during aminolysis of cephamycin and a consecutive reaction mechanism have been suggested on the basis of a carbon-13 NMR study[144].

4.1.2.5 Attack by the α-amino group of the 7-amido side chain of cephalosporin

Cephalosporin derivatives with α-amino substituents in their C-7-amido side chain, such as cephalexin, cefadroxil, cephradine, and cefatrizine, undergo intramolecular attack by the α-amino group on the β-lactam carbonyl carbon with cleavage of the β-lactam ring and formation of stable δ-lactams of the diketopiperazine type 4.20, (Scheme 4.18) :



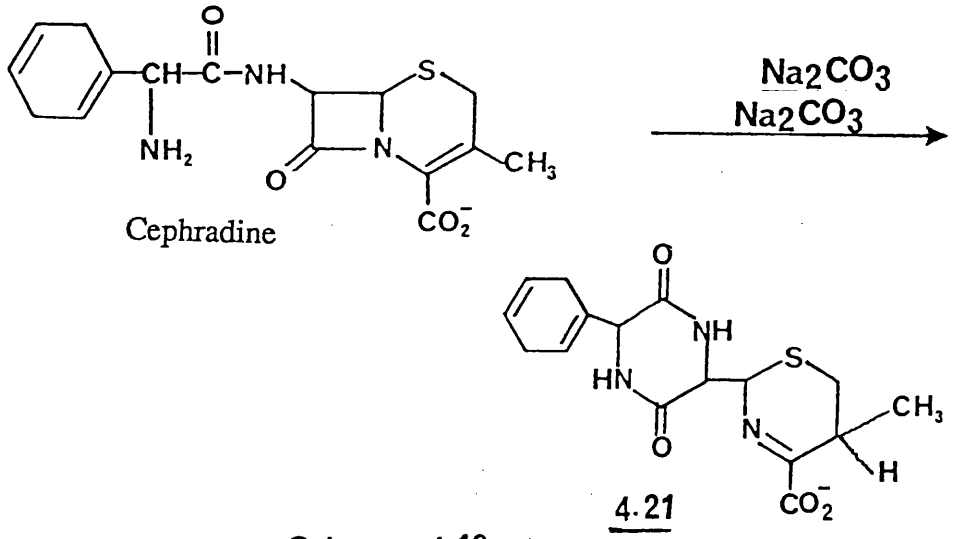
4.20

Scheme 4.18

This route has been established in several cases by isolation of diketopiperazines of type 4.20, and, in one case, an analogue with a 4-5 double bond 4.21[74] (p.129). No product of this kind has, however, been isolated from simple degradation media.

From an aqueous sodium carbonate solution of cephradine, Cohen et al. (1973)[74] isolated the diketopiperazine-type compound, 4.21, as the major alkaline degradation product (Scheme 4.19, p.129). The authors demonstrated the $\Delta^{4,5}$-double bond migration in the

degradation of cephradine from NMR observations (Scheme 4.19).



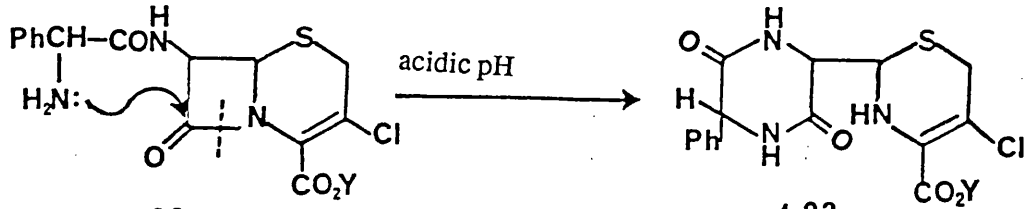
Scheme 4.19

Yamana and Tsuji (1976)[36] followed the degradation of cephalexin by NMR spectroscopy according to Cohen et al.[74] and by HPLC. In deuterium oxide-sodium deuteroxide (D_2O-NaOD) at pD 8.0 and 60°C, the 3-methyl group in cephalexin showed a singlet at δ 1.84, which gradually decreased in intensity as the degradation proceeded, and a new singlet for a secondary methyl group was observed at 1.22 ppm. When the degradation was performed in water at pH 8.0, the methyl proton signal appeared as a doublet (J=8.0 Hz). The authors suggested that, these NMR spectral changes for cephalexin indicated the double bond migration to the 4,5-position during the degradation and exhibited the same behaviour as for cephradine[74]. The HPLC studies also showed that, the chromatographic changes of the degraded solutions of cephalexin and cephradine at pH 8.0 exihibited the same elution pattern for all solutions.

Indelicato et al.[65,66] isolated the $\Delta^{3,4}$- diketopiperazine type, 4.20, from the refluxed benzene solution of the p-nitrobenzyl esters of cephalexin and cephaloglycin. The same authors[66], together with Bundgaard[73], and Yamana and coworkers[36,145] have demonstrated that intramolecular nucleophilic attack by the α-amino moiety on the β-lactam is possible conformationally in cephalosporin molecules, causing an apparent increase in chemical

reactivity and rate of hydrolysis of the β-lactam moiety, over compounds not containing an α-amino group in the 7-acylamido side chain. In contrast, penicillins with α-amino substituent, such as ampicillin, did not exhibit such a rate enhancement of the β-lactam opening by the direct intramolecular attack of the side-chain α-amino group[36]. This was attributed to the steric hindrance of the gem-dimethyl group and the 3-proton to the attack of the α-amino group at the β-face as suggested by Indelicato et al.[65,66].

Dinner et al. (1977)[146] attempted to isolate products from basic aqueous degradation of cefaclor 4.22a without success. However, a piperazine-2,5-dione 4.23a was isolated from the acidic aqueous degradation of cefaclor[67], and they obtained 4.23b from the refluxed benzene solution of the p-nitrobenzyl ester of cefaclor 4.22b[66], (see Scheme 4.20 below).



4.22 4.23

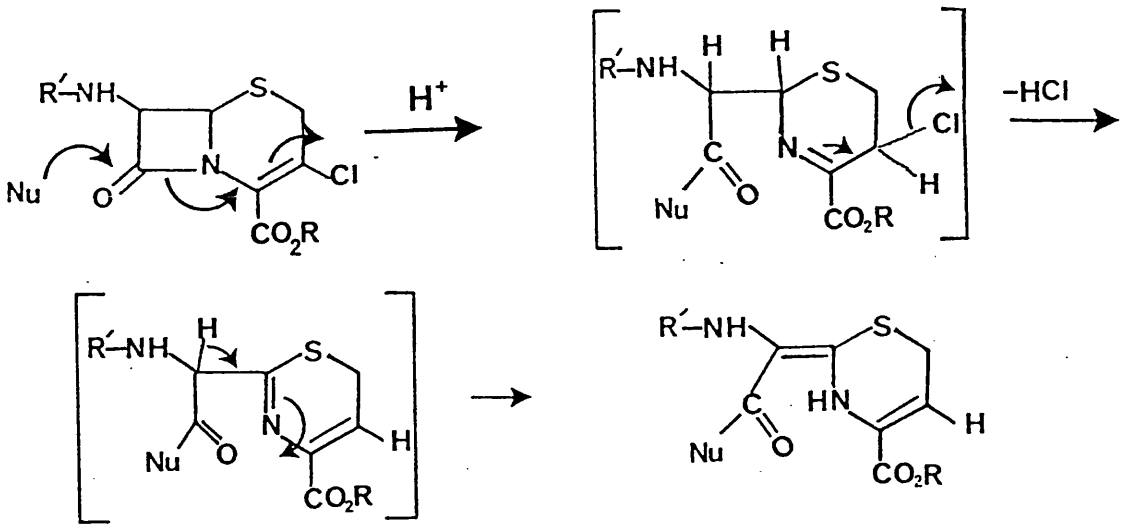
a: Cefaclor Y = H

b: Cefaclor-4-p-NB ester Y = $-CH_2-C_6H_4-NO_2$

Scheme 4.20

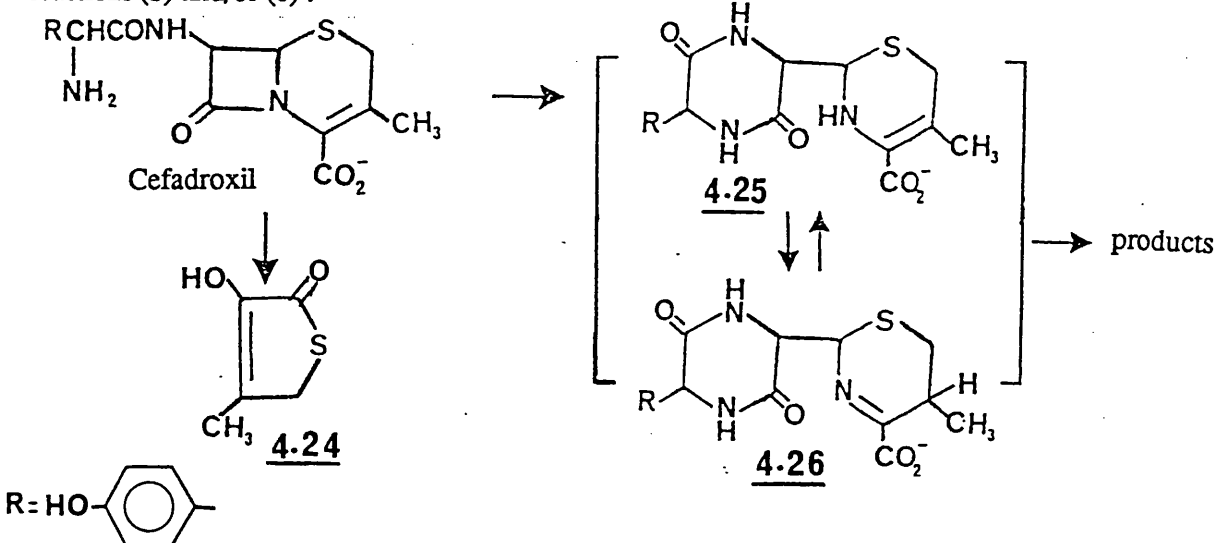
However, elemental analysis of 4.23b indicated the absence of chlorine. The peak assignments for the ^1H and ^{13}C spectra (in DMSO-d_6) of 4.23b indicated a hydrogen substituted for chlorine. The authors[146] proposed the following Scheme 4.21 (p.131) to account for a nonconcerted loss of chlorine subsequent to opening of the β-lactam, either

intra-molecularly by an α-amino moiety or by an external nucleophile :



Scheme 4.21

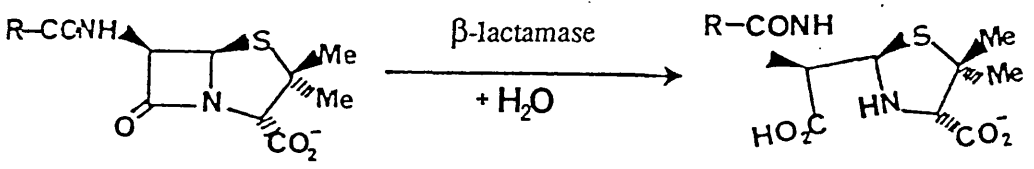
Tsuji and coworkers (1981)[64] described the degradation kinetics and mechanisms of cefadroxil, in aqueous solution at pH 2.51-11.5 at 35°C and ionic strength 0.5. The degradation rates were determined by high-pressure liquid chromatography (HPLC). The compound was shown to degrade by three parallel reactions : a) intramolecular aminolysis by the C-7 side-chain amino group in the β-lactam moiety, b) water-catalysed or spontaneous hydrolysis, and c) β-lactam cleavage by the nucleophilic attack of hydroxide ion. In neutral and weak alkaline solutions, the main degradation products were two piperazine-2,5-diones 4.25 and 4.26 and 3-hydroxy-4-methyl-2(5H)-thiophenone 4.24 (see Scheme below), the former being formed from reaction (a), while the latter arose via the degradation pathways of reactions (b) and/or (c) :



The structural elucidation of 4.24, 4.25 and 4.26 was based on spectral data. The ^{1}H NMR spectrum of 4.24 showed three singlets at 2.09, 3.74, and 6.10 ppm; those of 4.25 and 4.26 revealed two signals ascribable to methyl groups at 1.58 and 2.23 ppm in the reaction mixture, suggesting the existence of two different methyl groups, one attached to a secondary carbon atom and the other attached to a double bond (the former coupling with a vicinal proton). Signals at 4.05 and 5.41 ppm were assigned to the C-7 and C-6 protons, showing long-range coupling with protons on C-2 and/or C-3.

Notes on β-lactamases

β-lactamases are enzymes of bacterial origin which hydrolyse the C-N bond in the β-lactam ring of a penicillin or a cephalosporin[134]. A classification of these enzymes from gram- negative bacteria into five main classes containing 15 different types of enzymes was proposed[147]. Some β-lactamases are highly species specific, such as those found in all strains of *Pseudomonas aeruginosa*[41], whereas others, being mediated by an R factor such as R_{TEM}, may be found in several different species[148]. Some enzymes inactivate cephalosporins very much more rapidly than they inactivate penicillins. Usually the activity and the concentration of these enzymes are primarily estimated from their effect on a known concentration of benzylpenicillin and/or cephaloridine. Methods for assaying β-lactamase activity can be divided into two main groups. In one, the concentration of the unchanged substrate remaining is measured; in the other, the assay determines the concentration of breakdown products. In general, when the β-lactam ring of a penicillin is hydrolysed by a β-lactamase (Scheme 4.22 below), the corresponding penicilloic acid, which is a stable substance, is produced in stoichiometric proportions.



A penicillin

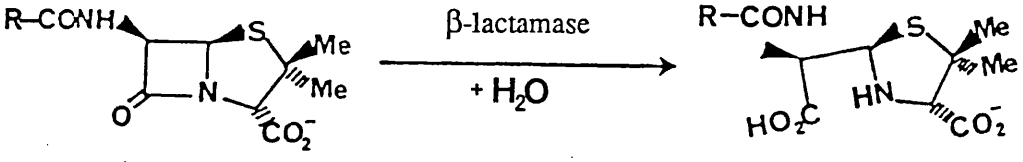
A penicilloic acid

Scheme 4.22

The situation with cephalosporins is more complicated owing to the presence of another substituent on the nucleus at position 3. The first product of β-lactamase attack on a cephalosporin is hypothetically a cephalosporanic acid (Scheme 4.23, p.134), analogous to

Notes on β-lactamases

β-lactamases are enzymes of bacterial origin which hydrolyse the C-N bond in the β-lactam ring of a penicillin or a cephalosporin[134]. A classification of these enzymes from gram- negative bacteria into five main classes containing 15 different types of enzymes was proposed[147]. Some β-lactamases are highly species specific, such as those found in all strains of *Pseudomonas aeruginosa*[41], whereas others, being mediated by an R factor such as R_{TEM}, may be found in several different species[148]. Some enzymes inactivate cephalosporins very much more rapidly than they inactivate penicillins. Usually the activity and the concentration of these enzymes are primarily estimated from their effect on a known concentration of benzylpenicillin and/or cephaloridine. Methods for assaying β-lactamase activity can be divided into two main groups. In one, the concentration of the unchanged substrate remaining is measured; in the other, the assay determines the concentration of breakdown products. In general, when the β-lactam ring of a penicillin is hydrolysed by a β-lactamase (Scheme 4.22 below), the corresponding penicilloic acid, which is a stable substance, is produced in stoichiometric proportions.

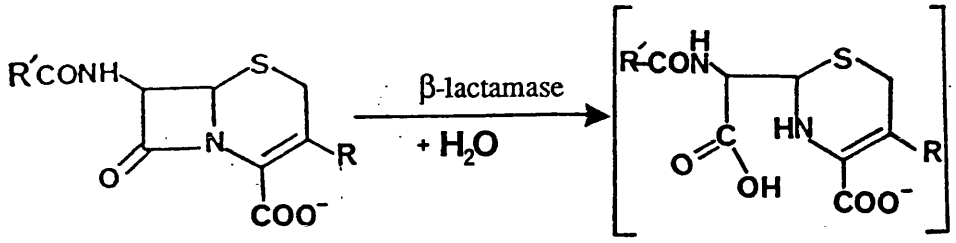


A penicillin — A penicilloic acid

Scheme 4.22

The situation with cephalosporins is more complicated owing to the presence of another substituent on the nucleus at position 3. The first product of β-lactamase attack on a cephalosporin is hypothetically a cephalosporanic acid (Scheme 4.23, p.134), analogous to

penicilloic acid, but usually very unstable and cannot be isolated as it undergoes rapid decomposition.



A cephalosporin

unstable intermediate

Scheme 4·23

Breakdown products

For a comprehensive account of β-lactamase enzymes the reader is directed to reference 149.

4.2 Aim of present work

Since the ^1H NMR technique yields detailed information about the degradation of β-lactam antibiotics of the penicillin class, we attempted to use the procedure to study the degradation of cephalosporins under various experimental conditions, i.e., alkaline hydrolysis, enzymatic hydrolysis, hydrazinolysis and acidic degradation. In the next section we describe the results of the ^1H NMR study and endeavour to determine the structures of the degradation products with reference to previous studies in the field.

To complement the work with enzymes, the value of ^1H NMR spectroscopy in monitoring the action of β-lactamases on a group of penicillin antibiotics was also carried out. The results are presented separately on page **177** as a supplement to this Chapter.

4.3 Experimental

4.3.1 Instrumentation

1. The ^1H NMR spectra were recorded on a Jeol FT NMR GX270 spectrometer already described in Chapter three (section 3.3.1).

2. Readings of pH were carried out on a Philips PW 9410 digital pH meter.

4.3.2 Materials

1. 7-phenylacetamidodesacetoxycephalosporanic acid was obtained as described under its method of preparation (Chapter two, section 2.2.3.5).

2. Sodium salts of benzylpenicillin, cephalothin, cefoxitin, cephaloridine, cefuroxime, cefotaxime, cephazolin, diammonium salt of moxalactam, cefaderoxil, cefaclor, cephradine and cefatrizine, were obtained as described in Chapter two (Table 2.1). The compounds were used as received.

3. 99.8 % D_2O was purchased from Aldrich, England.

4. Hydrazine hydrate, 99% ($NH_2NH_2.H_2O$) was obtained from Fluka AG chemicals, W.Germany.

5. NaOD (40 % in D_2O), Goss Scientific Instruments, England.

6. β-Lactamase enzymes; two types were used :

a) β-lactamase (ex. Bacillus cereus 569/H9), broad spectrum mixture, obtained from Genzyme Biochemicals, Kent, England.

and b) broad spectrum β-lactamase mixture, obtained from Centre of Applied Microbiology and Research, Porton Down, Salisbury, England.

Both enzymes were stored at -5°C.

7. Deionised water was used where necessary.

8. Carbonate and phosphate buffers prepared as follows :

0.2 M phosphate buffer of pH 9.6 (pD 10.0) :

0.2 M deuterated Na_2HPO_4	10 ml
0.25 M deuterated Na_3PO_4	a quantity enough to adjust pH to 9.6.

0.25 M Carbonate buffer pH 9.6 : Prepared by addition of 0.25 M deuterated Na_2CO_3 to 0.25 M $NaHCO_3$ to adjust pH to 9.6.

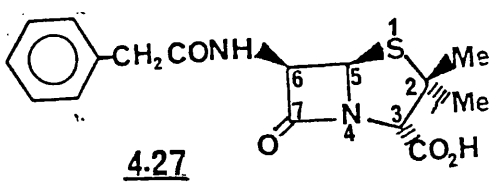
4.4 Results and discussion

4.4.1 Penicillins

4.4.1.1 Direct ^{1}H NMR observations on hydrazinolysis of benzylpenicillin Na 4.27

Literature results

Review of the available literature at the time revealed lack of any direct ^{1}H NMR study on the hydrazinolysis of benzylpenicillin. The only work of interest is that of Hamilton-Miller et al.[44a] on the aminolysis of benzylpenicillin. The changes in chemical shifts before and after the reaction are shown in Table 4.1 (p.137).



4.27

Table 4.1 : Chemical shifts before and after aminolysis of benzylpenicillin[a] :

Substance	6-H	5-H	3-H	α,β-2-Me	$ArCH_2$
Intact	5.52 d (4.0)	5.43 d (4.0)	4.23s	1.55s, 1.49s	3.64s
product	4.98 d (7.5)	4.38 d (7.5)	3.45s	1.55s, 1.23s	3.68s

a s=singlet, d=doublet.

Of particular interest are the upfield shifts of the β-lactam proton signals, accompanied by an increase in coupling constant. There was also an upfield shift in the signal from the C-3 proton and a slight downfield shift in the signals from the side-chain methylene protons. The authors reported that the spectrum became considerably more complicated after 24 hours, indicating that more than one species was present in the reaction mixture and suggested that epimerization had occurred at C-5. The structures of these degradation products were not determined, and no mechanism was suggested for their formation. (see our results with hydrazine/benzylpenicillin below).

1. Preparation of ^1H NMR sample

10 mg of benzylpenicillin Na salt 4.27 was dissolved in 0.5ml D_2O and 5 µl hydrazine hydrate (neat) was added. ^1H NMR spectra at room temperature (~ 24°C) were taken immediately and subsequently at appropriate intervals. The last three spectra (at day 8, 13, and 37) after storage at 37°C.

2. Results and discussion

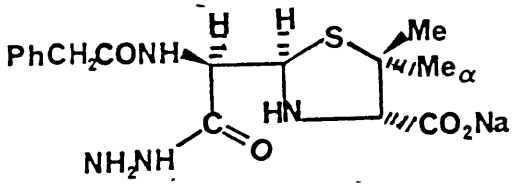
The initial spectrum (Fig. 4.3a, p.139), recorded 5 minutes after the start of the experiment, showed evidence of rapid β-lactam ring opening, indicated by the complete

absence of the intact 5-H/6-H signals (5.50, 5.40 ppm), accompanied by the appearance of a novel upfield set of two doublets centered at 4.92, 4.27 ppm with increased coupling constant, 8.8 Hz. There was also an upfield shift of the C-3 proton signal (4.23 ppm moved to 3.43 ppm) and the α-2-Me signal (1.49 ppm to 1.21 ppm). Furthermore, the AB quartet centered at 3.64 ppm, assigned to the non-equivalent benzylic CH_2 protons of benzylpenicillin, changed to an intense singlet at 3.65 ppm.

80 minutes later, the high-field region of spectrum (Fig. 4.3b, p.139) showed two extra lines at 1.88 ppm and 1.79 ppm, each integrating to about one proton. No other change was observed.

Fig. 4.3c shows the spectrum of the reaction mixture 24 hours after the start of the experiment. Little change occurred, apart from increases in intensity of the singlets at 1.88, 1.79 ppm (all the signals in the 1.90 - 1.20 ppm region had the same intensity, i.e., 1.88, 1.79, 1.54 and 1.21 ppm signals) and the appearance of minor peaks at 1.50 - 2.50 ppm and 3.40 - 3.80 ppm regions.

The subsequent spectra, run 2, 5, 6 and 8 days later show insignificant changes, apart from the increased intensities of some minor peaks at 1.30 ppm(~ 0.4 H) and 3.71 ppm (~ 0.4 H); the 5-H signal at 4.26 ppm weakened (~ 0.7 H), and the singlet at 3.42 ppm integrated to about 0.7 H. These results may be interpreted in terms of rapid opening of the β- lactam ring with formation of the hydrazide 4.28.



4.28

Changes in signals due to 3-H, 5-H, 6-H and α-2-Me are remarkably close to those of the aminolysis product (Table 4.1) even in regard to the increased ^3J value between 5-H and 6-H. The extra signals at 1.88 and 1.79 ppm cannot be explained at present. They are unlikely to arise from 2-Me signals of an epimeric form of 4.28 because integrals of the 1.55 and 1.21

ppm singlets do not fall below 3 protons after appearance of the new signals. In addition, no other signals diagnostic of epimerization appear.

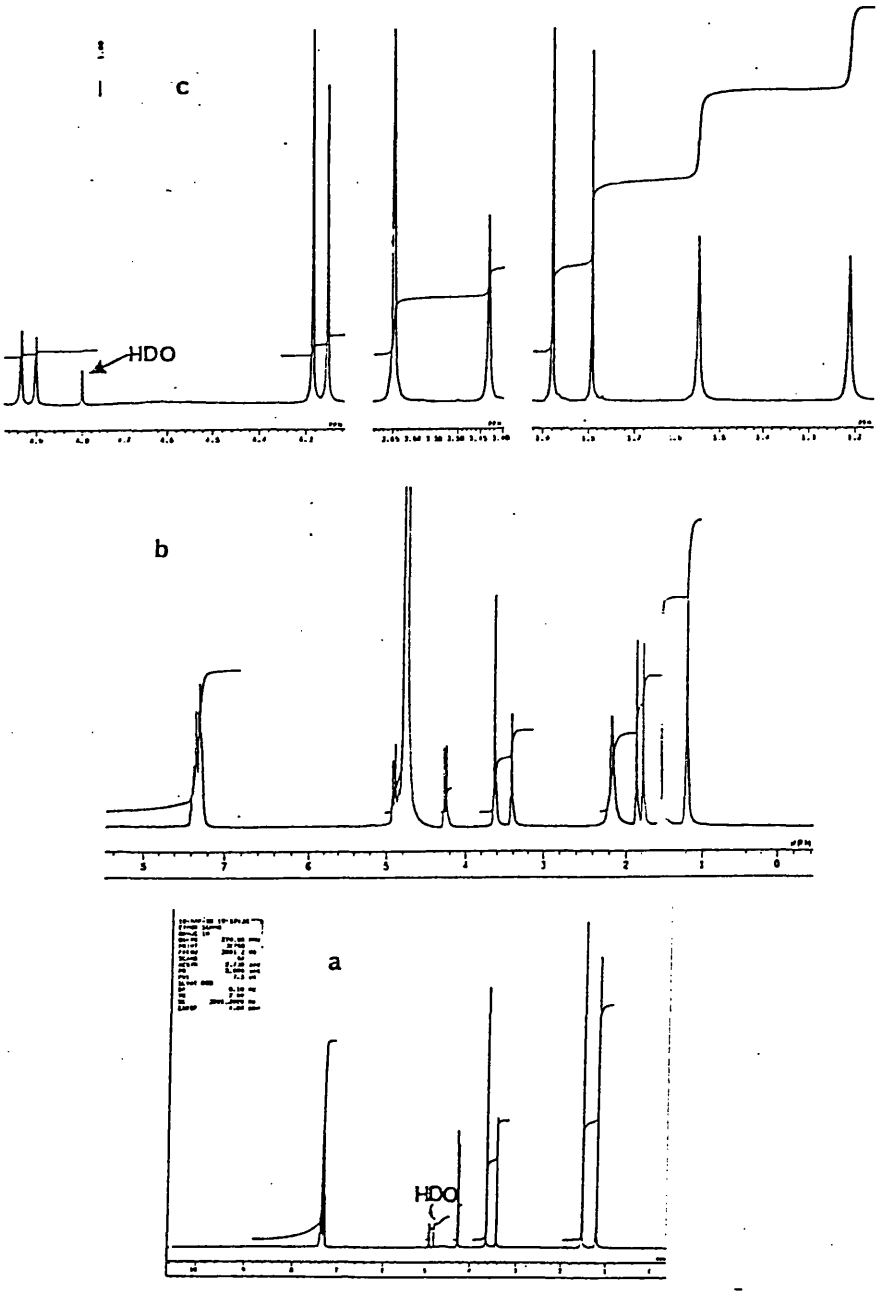
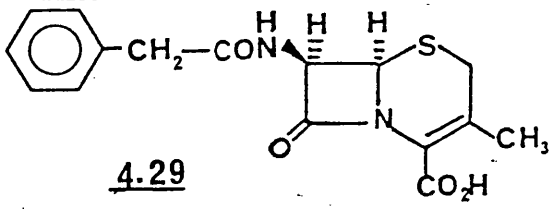


Fig. 4.3 : 270 MHz ^{1}H NMR spectra of the hydrazinolysis of benzylpenicillin Na. a: Initial (5 min), b: 80 min and c: 24 hrs after commencement. Integral distortions in (a) and (c) spectra due to suppression of HDO signal. See text for details.

4.4.2 Direct ^{1}H NMR observations on the degradation of cephalosporin antibiotics

4.4.2.1 Degradation work on 7-(2-phenylacetamido)desacetoxy-cephalosporanic acid 4.29



4.29

1. Reasons for choice of 4.29

The 7-(2-phenylacetamido) derivative of desacetoxycephalosporanic acid 4.29 was selected as the chief compound for degradation investigations for the following reasons :

(i) It is a close cephalosporin analogue of benzylpenicillin.

(ii) It has a non-leaving substituent at position-3.

(iii) Absence of an amino substituent to the 7-amido side chain (to avoid formation of ketopiperazine products).

2. Source of 4.29

Literature search

Two methods were described in literature[152] for the synthesis of 4.29 from benzylpenicillin sulphoxide.

The first method involves the heating of a mixture of the sulphoxide of benzylpenicillin, trimethylchlorosilane and α-picoline in chloroform for 20 hours at 83°C; after cooling the mixture was stirred with water and the pH adjusted to 7.5 with 4N KOH solution. The aqueous layer was separated, pH adjusted to 1.5 with 4N HCl, and extracted with ethylacetate. The organic solvent was then replaced by 1-propanol and cooled to ~ 0°C. Addition of water and a 1.25 M solution of potassium salt of 2-ethylcaproic acid in butylacetate gave a precipitate, which after filtering and drying, contained the potassium salt of Δ^3-7-phenylacetamidodesacetoxycephalosporanic acid (yield 47%), in addition to a minor yield of the Δ^2-isomer.

The second method involves the reaction of the sulphoxide with dioxane, N,O-bis(trimethylsilyl)acetamide (BSA), α-picoline and a solution of α-picoline hydribromide in dichloromethane yielding ~ 78% of the potassium salt of Δ^3-7-phenyl-acetamidodesacetoxycephalosporanic acid.

Material used in this work was obtained from the corresponding p-nitrobenzyl ester kindly supplied by Glaxo Laboratories. Details are given in Chapter two (section 2.2.3.5).

3. Results and discussion

(A) Effect of buffers alone

The ^{1}H NMR chemical shifts of the spectra of reaction mixtures of 4.29 in either 0.25 M deuterated sodium carbonate buffer (pD 10.0) or 0.1M deuterated phosphate buffer of pH 9.0, showed no indication of β-lactam ring opening, even after 4 hours. Apart from extra minor signals in the 3-Me region (1.90-1.80 ppm), negligible change was observed, suggesting that 4.29 is stable in buffered solutions over the pH range 9.6 - 10.0.

(B) ^{1}H NMR analysis of products of 7-phenylacetamido-derivative 4.29 hydrolyzed in carbonate buffer in the presence of β-lactamase

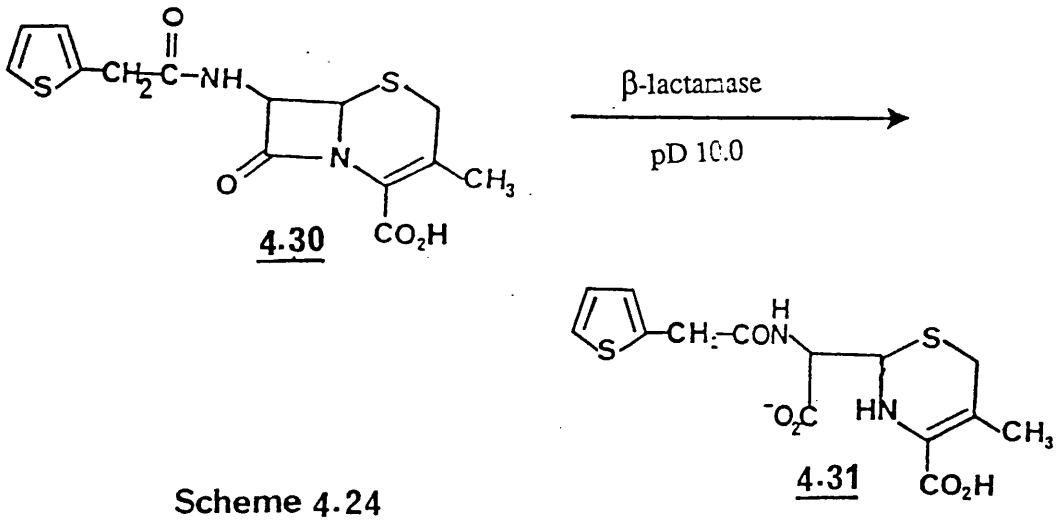
1. Preparation of ^{1}H NMR samples

7-(2-phenylacetamido)-desacetoxycephalosporanic acid 4.29 (6.0 mg) was dissolved in 0.5 ml 0.25 M deuterated sodium carbonate buffer at pD 10.2
and either a) few crystals of β-lactamase (Porton),
or b) few crystals of β-lactamase (Genzyme),
was added.

^{1}H NMR spectra were taken immediately and subsequently at appropriate intervals (solution stored at 5°C).

2. Results and discussion

Pratt et al. (1986)[153] followed the hydrolysis of desacetoxycephalothin 4.30, a close analogue to 4.29, in 0.25 M Na_2CO_3 buffer (pD 10.0) in presence of either R_{TEM-2} (see p.133) or the P99 β-lactamase, by ^1H NMR. They observed instant changes (< 1.0 min) in the initial spectrum to that of the ring opened product 4.31, as indicated by the upfield movements of the C-6 and C-7 proton signals of the intact antibiotic (Scheme 4.24, Table 4.2 below), and little change in the C-3' signal :



4.30 4.31

Scheme 4.24

Table 4.2 : Chemical shifts (ppm) before and after enzymic hydrolysis of 4.30

	6-H/ 7-H	3-Me
4.30 (intact)	5.07, 5.59 d	1.90 s
4.31 (enzymatic product)	4.51, 4.59 d	1.84 s

No detailed ^1H NMR data of product 4.31 were described to support its structural identity; also no mention of the presence of epimers was reported.

a) Hydrolysis of 7-phenylacetamido-derivative 4.29 in pD 10.2 + Porton enzyme

The ^1H NMR spectra of the degradation mixtures were analysed by observing any notable changes in the 2-CH_2, 3-Me, and 6-H/ 7-H signals of the products and by comparison with the ^1H NMR spectra of 4.29 (intact) in D_2O.

The initial ^1H NMR (Fig. 4.4a, p.144), run immediately after buffer and β-lactamase addition, showed the complete absence of the 6-H/ 7-H doublets of the intact molecule at 5.05 and 5.56 ppm, and appearance of new well resolved doublets centred at 4.52 and 4.60 ppm (J ~ 5 Hz), corresponding to the C-6 and C-7 protons of the β-lactam ring opened product. Also evident were a second pair of doublets which flanked the HDO signal, centred near 4.72 and 4.86 ppm (J ~ 3 Hz), i.e.,

	6-H	7-H	J in Hz
Intact molecule of 4.29	5.05 d	5.56 d	4.5 (cis)
Ring opened product	4.52 d	4.60 d	5.0 (cis)*
(enzymatic hydrolysis)	4.72 d	4.86 d	~ 3.0

* Small separation change is evidence that the 7-H, 6-H configurations are retained.

The chemical shifts of C-6/ C-7 protons of desacetoxycephalothin[153] (p.142) and those of the analogous protons of benzylpenicillin (5-H, 6-H)[85] also moved upfield when the β-lactam rings opened. The rest of the spectrum, Fig. 4.4a, showed little change apart from the slight upfield shifts of the two doublets due to 2-CH_2 and the appearance of two (1:2) singlets at 1.90 ppm (sharp) and 1.86 ppm (broad) with an integration (total)= 3 protons.

A spectrum recorded 90 min after the start of the experiment was similar to the initial spectrum and differed only in the appearance of fine structure on the lower field 2-CH_2 doublet and a small change in the relative areas of the two signals near 1.90 ppm.

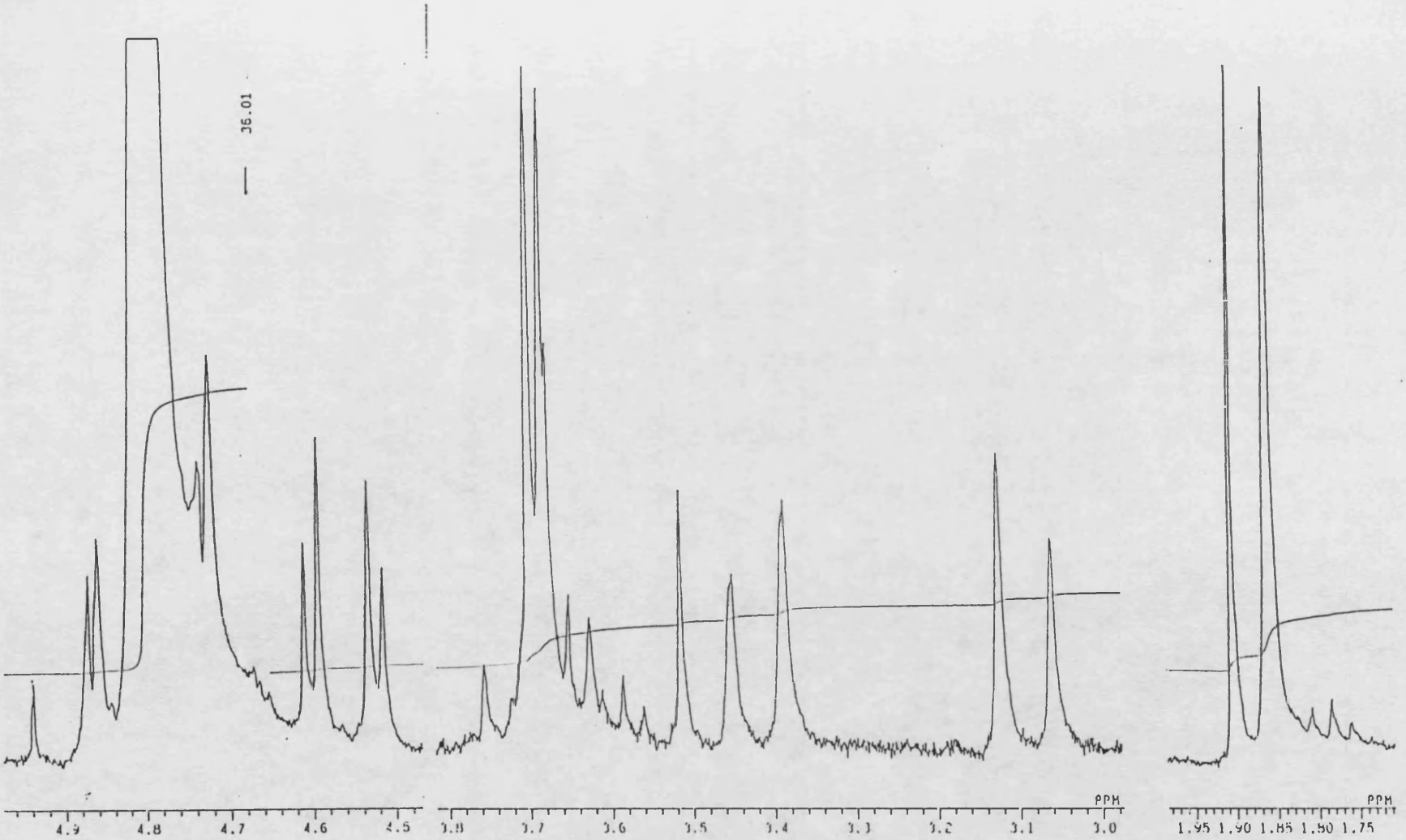


Fig.4.4a : Part of the 270 ^{1}H NMR spectrum of the 7-phenylacetamido derivative 4.29 in buffer pD 10 + β-lactamase (Porton) recorded immediately after preparation. The broad band centred near 4.8 ppm is due to HDO.

Eighteen hours later, the following changes were observed in the spectrum (Fig.4.4b, p.146) :

i) the region close to the HDO band now showed a doublet pair centred near 4.55 and 4.66 ppm in addition to the 4.52/ 4.60 ppm and 4.72/ 4.86 ppm doublets (these new signals were also apparent at low intensity in the 90 min spectrum),

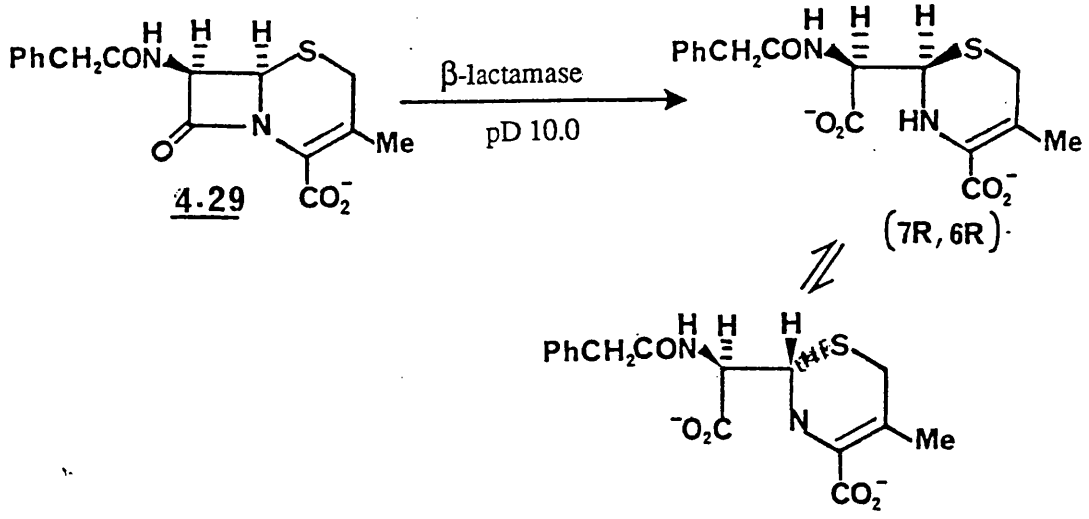
ii) both halves of the 2-CH_2 signal were split into overlapping doublets,

iii) the $ArCH_2$ signal also split into two overlapping doublets,

iv) the 3-Me region (1.90 - 1.85 ppm) now showed three lines : two closely placed lines at 1.90 - 1.91 ppm and a broad singlet at 1.86 ppm, with 1:2 relative intensities.

Nine days after the start of the experiment, the reaction mixture spectrum, was even more complex.

Although spectra could not be analysed in detail, since reference products of defined NMR parameters were not available, the results are clear evidence of enzyme-induced opening of the β-lactam ring and the rapid isomerization of the 7-R, 6-R-analogue of benzylpenicilloic acid. After 90 minutes, signals due to a third product were evident, clearly resolved after 18 hours. (Scheme 4.25) :



Scheme 4.25

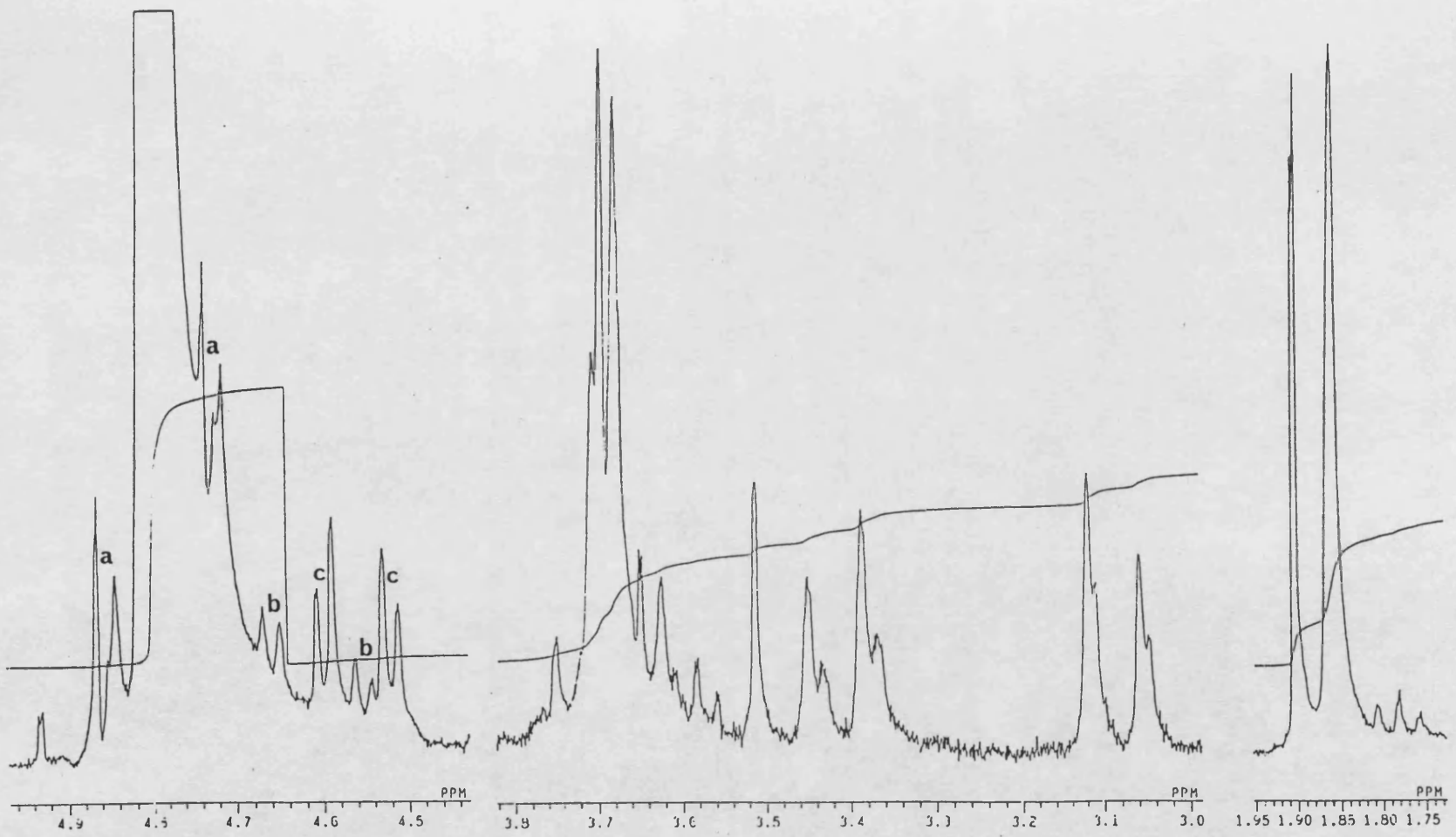


Fig.4.4b : As for Fig.4.4a but recorded 18 hours after the start of the experiment. The three doublet pairs in the 7-11, 6-11 region are designated a/a, b/b, and c/c.

b) Hydrolysis of 7-phenylacetamido-derivative 4.29 in pD 10.0 + Genzyme β -lactamase at 5°C :

The initial spectrum, showed little apparent change except for a new minor signal close to 3-Me signal at 1.90 ppm.

An hour later, the 6-H, 7-H signals of the starting (intact) material 4.29 were still present but of reduced intensity, plus a new pair of doublets centred at 4.60 and 4.52 ppm (separation 4.8 Hz). In the 2-CH_2 region of the spectrum, doublets due to the intact antibiotic were present (centred near 3.18 and 3.55 ppm) plus lower intensity doublets to higher field (centred near 3.08 and 3.42 ppm) as seen in the spectrum of material treated with the Porton enzyme. Three signals were displayed in the methyl region, one major at 1.90 ppm with a minor splitting, and a new broad signal at 1.86 ppm, having a 3-proton integration.

The following was observed in the spectrum recorded 17 hours after commencement :

i) The 5.55, 5.05 ppm doublets, due to the 7-H/ 6-H of the starting compound were absent.

ii) The 4.60, 4.52 ppm doublets were of increased intensity accompanied by a new set of low intensity doublets centred near 4.55 and 4.66 ppm (the 4.72/ 4.86 ppm signals of the Porton spectrum could not be resolved, possibly obscured by the HDO signal and side bands).

iii) The 3-Me region showed two signals : one narrow at 1.90 ppm (no splitting), and the other broad at 1.86 ppm, with an intensity of 3:5, respectively.

iv) In the 2-CH_2 region (3.60-3.00 ppm), the 3.55, 3.19 ppm doublets, due to the 2-CH_2 of the starting material were absent; two sets of signals centred at 3.40, 3.10 ppm appeared overlapping, with the lowfield doublets showing fine splitting.

In a spectrum run after a 9 day period all these features were apparent but with changes in relative intensities, Table 4.3, p.148).

Table 4.3[a] :

	7-H,6-H	2-CH_2	3-Me
Opened ring products	4.60, 4.52 d (4.8)	dd centred 3.40 (18)	1.86 s,
	4.67, 4.56 d (4.6)	dd centred 3.10 (18)	1.91 s

a s=singlet, d=doublet, J values in parenthesis.

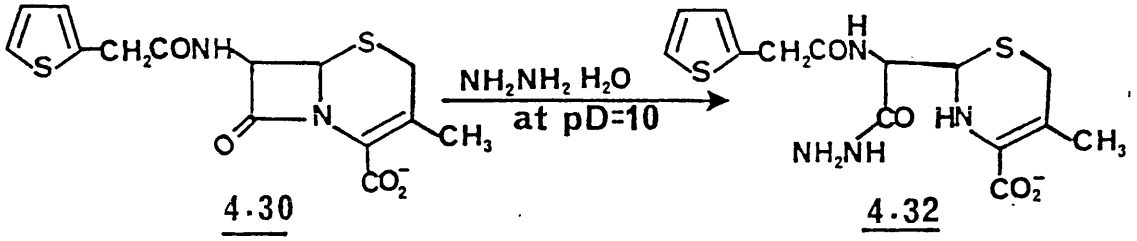
Thus results comparable to those observed after use of the Porton enzyme were found.

Under the conditions of these experiments in which there was little control over quantities of enzymes employed, the Genzyme product was slower acting than the β-lactamase obtained from Porton.

(C) ^{1}H NMR observations on the hydrazinolysis of the 7-phenylacetamido derivative 4.29

Pratt and Faraci (1986)[153] examined the reaction of hydrazine with desacetoxycephalothin 4.30 (and other cephalosporin derivatives) at pH 9 and 5°C. UV spectra showed a hydrazine-catalysed, rapid (< 10 s) absorbance decrease at 260 nm, of similar magnitude to that observed in the β-lactamase-catalysed hydrolysis. Absorbance at λ260 nm is characteristic of intact cephalosporins. The authors followed the reaction by ^{1}H NMR experiments and the initial spectrum changed instantly (< 1 min) to that of 4.32 (presumed) whose upfield movements of the C-6 and C-7 protons chemical shift support this structural assignment (Table 4.4, p.149). They interpreted their results in term of Scheme 4.26 reaction pathway. No detailed ^{1}H NMR analysis of 4.32 was described to support

this degradation Scheme :



Scheme 4.26

Table 4.4 :

	6-H/ 7-H[a]	3-Me
Intact 4.30	5.07, 5.59 d	1.90 s
Hydrazinolysis product 4.32	4.55d, obscured	1.87 s
Enzymatic product 4.31	4.51, 4.59 d	1.84 s

a (no separations reported); d=doublet, s=singlet.

Results and discussion :

The changes in ^{1}H NMR spectra that occurred when 4.29 was allowed to react with hydrazine hydrate in presence of a deuterated buffered solution of 0.1 M sodium carbonate (pD 10.0) are shown below.

The starting spectrum of the reaction mixture, Fig.4.6a (p.151, 10 min later), showed the following significant changes :

i) The 6-H/ 7-H signals of the starting compound were still present, but with much reduced intensity, accompanied by the appearance of a set of two doublets in the 4.40-4.65 ppm

region of separation ~ 9 Hz.

ii) Appearance of a new sharp singlet (integrated for about one proton) near 4.50 ppm.

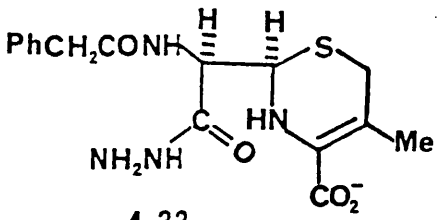
iii) The methyl signal split into 3 lines (with a 3 proton integration) at 1.89 ppm (sharp), 1.86 ppm (broad, major) and 1.84 ppm.

iv) The 2-CH_2 signal shifted upfield, the outer peaks greatly weakened.

165 minutes later, the original signals from the β-lactam protons had virtually disappeared, and the two doublets centred at 4.45, 4.61 ppm (J=9.3 Hz) were now well resolved, except for the intensity of the lowfield doublet being slightly affected by the water broad peak centred near 4.80 ppm, and integrating for about 2 protons (Fig.4.6b). The disappearance of the original 2-CH_2 AB system (centred at 3.19, 3.56 ppm) and its replacement by two sets of overlapping double doublets centred at 3.09, 3.23 ppm (which still integrated for about 2 protons) seemed to be synchronous with the disappearance of the original 6-H/ 7-H signals. The $ArCH_2$ signal shifted slightly upfield and showed signs of further splittings. The three lines in the methyl region at 1.89 ppm (narrow), 1.86 ppm (broad) and 1.83ppm (broad) had relative intensities of 1:2:2, respectively.

The spectrum was much the same after 24 hours.

These results indicate that the initial product of hydrazinolysis is a single isomeric form of the hydrazide 4.33 in that novel 2-CH_2, 6-H and 7-H ^1H resonances appear upfield of the original signals.



4.33

The ^3J value between 6-H and 7-H is notably greater than the value found in the intact antibiotic but comparable to the coupling found for equivalent protons of hydrazinolysis products of benzylpenicillin (see p.137). On storage further resonance signals appeared,

notably clear duplication of the 2-CH_2 signal (2 sets of AB type). This could be a result of epimerization of 4.33 at C-6 and C-7. Epimeric 6-H and 7-H doublets could not be resolved; perhaps these are obscured by the broad HDO signal near 4.8 ppm.

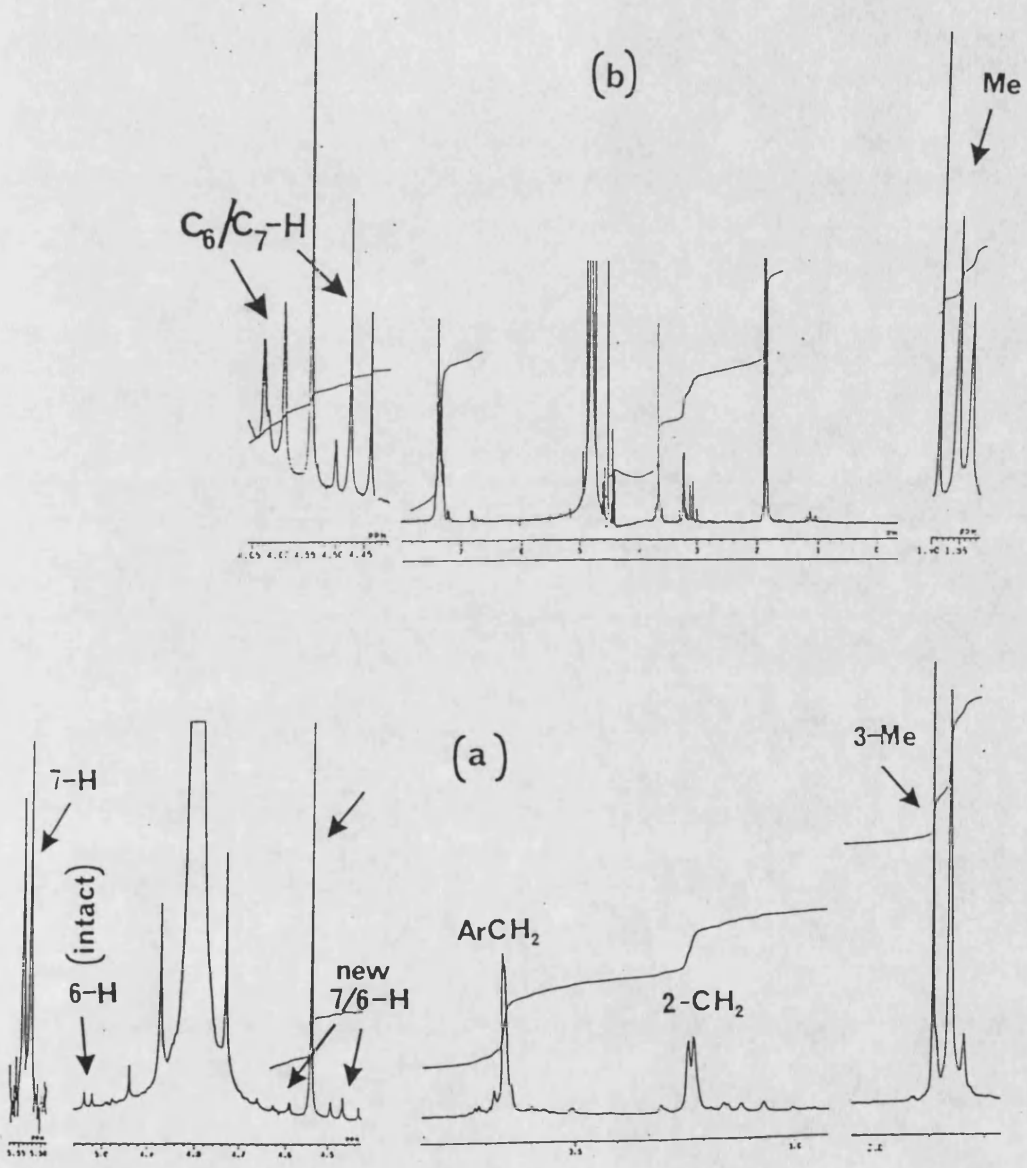


Fig.4.6 : Part of the 270 MHz ^{1}H NMR spectra of 7-phenylacetamido derivative 4.29 (in carbonate buffer pD 10) in presence of hydrazine hydrate :

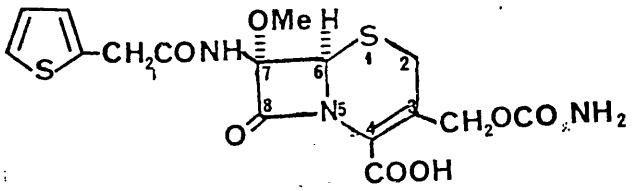
a) recorded immediately after preparation,

b) 165 min after the start of the experiment.

(See text for details).

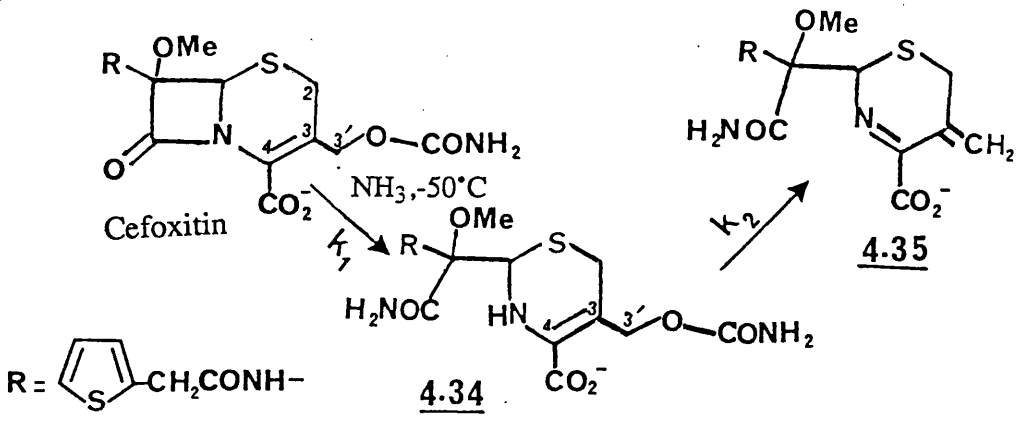
4.4.2.2 ^{1}H NMR study of degradation of cephalosporins with good leaving groups at C-3'

Cefoxitin



Literature results

Grabowski et al.(1985)[144] reported a ^{13}C NMR study of the aminolysis of cefoxitin (liquid ammonia at -50°C) which they claimed to be the first spectroscopic characterisation of the intermediate resulting from β-lactam cleavage (4.34) prior to loss of the carbamic acid anion from position 3' (4.35), i.e., a two-step process was proposed (Scheme 4.27, below) :



Scheme 4.27

The authors stated that the intermediate 4.34 had a lifetime sufficient for ^{13}C spectral characterisation. Subsequent reaction yields 4.35 which is stable to the aminolysis conditions, and readily characterised by carbon-13 spectroscopy. Identification of 4.34 and 4.35 was based on the ^{13}C data, and the chemical shifts of cefoxitin and its aminolysis products are listed in Table 4.5 (p.153).

Table 4.5 : ^{13}C chemical shifts (in ppm) of cefoxitin, 4.34 and 4.35 :

Carbon atom	Cefoxitin	4.34[a]	4.35[a]	Notes
C-2	26.05	28.39	30.04	
C-3	114.30	101.74	131.33	
C-4	134.05	139.60	172.09	
C-6	63.42	58.36	67.21	
C-7	95.43	89.38	87.73	
C-8	160.72	171.32	171.50	
C-3'	64.59	66.63	123.25	evidence of alkenic nature

a Numbering of 4.34 and 4.35 follows that of cefoxitin.

4.34 was identified by the increased shielding of C-3, now conjugated to an amine rather than an amide nitrogen, and large changes in shielding of C-4, C-6, C-7, and C-8, the latter having been converted to a primary amide carbonyl. Conversion to 4.35 produced some 30 ppm deshielding of C-3 and C-4. The exo-methylene carbon becomes olefinic (C-3') and appears downfield. Nevertheless, the structural identity of this intermediate has been questioned[154].

Discussion of present work results :

1. Enzymatic hydrolysis of cefoxitin :

From the ^1H NMR spectra of the reaction mixture of cefoxitin Na in 0.5 ml of deuterated water (D_2O) and few crystals of β-lactamase (Genzyme) at room temperature, run immediately and at appropriate intervals during 8 days of storage, the compound appeared to be substantially stable to enzymatic hydrolysis, as no signal changes occurred apart from appearance of minor peaks.

2. Hydrazinolysis reaction on cefoxitin Na :

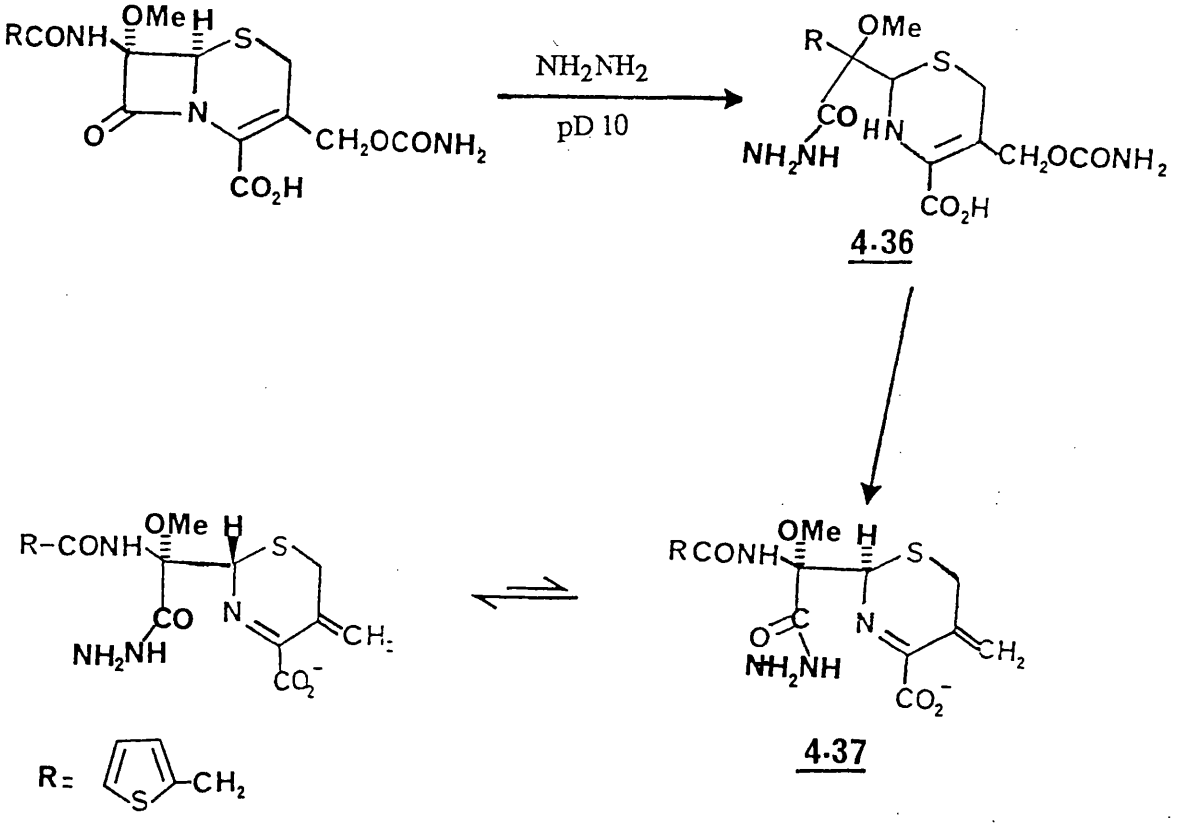
8 Mg of cefoxitin Na were dissolved in 0.5 ml 0.25M deuterated carbonate buffer solution at 5°C, then 5 µl hydrazine (neat) was added to the alkaline solution in an NMR tube. ^{1}H NMR spectra were taken immediately and subsequently at appropriate intervals (stored at 5°C).

The initial spectrum (Fig.4.7a, p.156), run 10 min after the start of the experiment, gives evidence of the rapid β-lactam ring opening, partial expulsion of the 3'-substituent and production of a structure with an exocyclic methylene group, indicated by the upfield shift of the signal due to the 7-OMe (3.52 ppm moved to 3.22 ppm), the much weakened double doublets at 4.64, 4.80 ppm and the appearance of two signals at 5.66, 5.71 ppm, with separation of 12.8 Hz similar to that of the non-equivalent 3-CH_2 protons attached to an sp^2 carbon atom; the downfield shift indicates the olefinic nature of the exo-methylene at C-3.

21 Hours later (Fig. 4.7b, p.156), the signals at 4.64, 4.80 ppm were competely absent; signals at 5.14 ppm (due to C-6), and 5.65, 5.70 ppm (C_3==CH_2) had slightly weakened. The 7-OMe, 2-CH_2 and ArCH_2 signal regions were more complicated, showing overlapping of peaks. The spectrum after 3 days of starting the experiment, was even more complex, showing appearance of new signals at 5.30, 5.40 ppm (as singlets), and a double doublet centered at 5.50 ppm. Signals at 5.14 ppm (s), 5.65 ppm (s) and 5.70 ppm were greatly weakened (trace only). Other signals, due to 7-OMe, 2-CH_2 and ArCH_2, were more complicated and difficult to interpret.

It is clear from the above ^{1}H NMR data of the initial spectrum of the reaction mixture, the cleavage of the β-lactam ring, and the intermediate 4.36 (Scheme 4.28, p.155) prior to loss of the carbamic acid anion from position 3', has enough lifetime for ^{1}H NMR to record the double doublet at 4.64, 4.80 ppm due to the residual 3-CH_2OCONH_2. Subsequent reaction yields the exo-methylene product 4.37 which is stable to the hydrazinolysis

condition for at least 20 hours, and readily characterised by ^{1}H NMR spectroscopy. Later, epimerization at C-6 appears to have occurred, and more than one epimer was present in the reaction mixture, as shown by the change in chemical shifts of signals due to 6-H and C3=CH_2 protons, and the overlapping of more than one set of peaks in the 7-OMe, 2-CH_2 and ArCH_2 regions.



Scheme 4.28

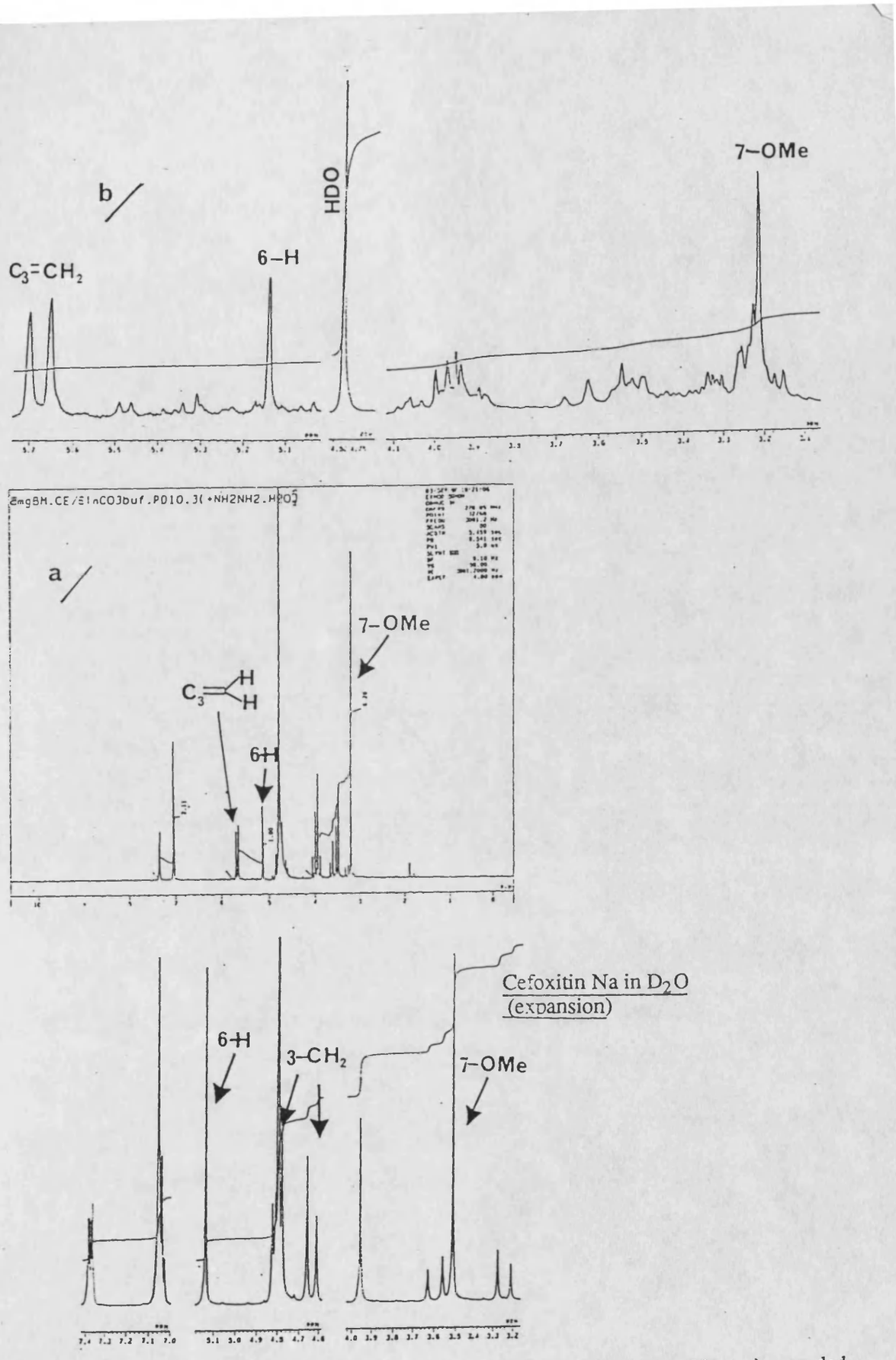


Fig. 4.7 : 270 MHz ^{1}H NMR spectra of the hydrazinolysis of cefoxitin Na : a) recorded immediately after preparation, and b) run 21 hrs after commencement. See text for details.

4.4.3 Effect of addition of NaOD-D_2O on cephalosporins (uncontrolled high pH)

4.4.3.1 Preparation of ^1H NMR sample

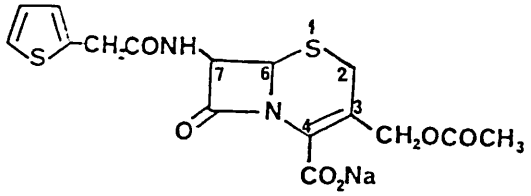
20 to 30 mg of the cephalosporin was dissolved in 0.5 ml D_2O at room temperature (24°C) and the ^1H NMR spectrum of the aqueous solution recorded. A few drops of deuterated sodium hydroxide (NaOD, 40 %) were then added to the aqueous solution in the NMR tube. ^1H NMR spectra (at 270 MHz) were then taken immediately and at appropiate intervals.

4.4.3.2 Results and discussion

a) Cephalosporins with a good leaving group at C-3

These materials generally produce complex spectra but in most cases signals diagnostic of exomethylene products may be resolved.

1. Cephalothin Na



A very complex spectrum resulted in presence of NaOD, but the following changes were noted :

i) disappearance of doublets due to 6-H, 7-H (centred at 5.10, 5.60 ppm, separation 4.9 Hz), and 3'-CH_2 (at 4.65, 4.82ppm, 12.5 Hz).

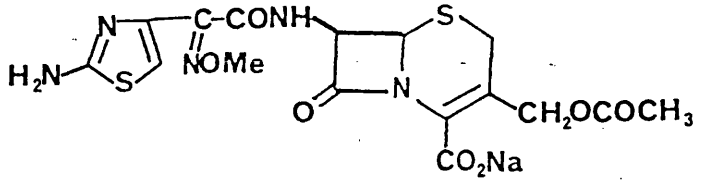
ii) low field shift of the $ArCH_2$ signals (dd centred at 3.86 ppm moved to 4.17 ppm, separation 16 Hz), and the upfield movement of the singlet due to OCOMe (from 2.06 ppm to 1.84 ppm), the latter position is characteristic of acetate.

iii) appearance of 3 doublets centred at 5.30, 5.40 and 4.58 ppm, with separations of 4.6 Hz.

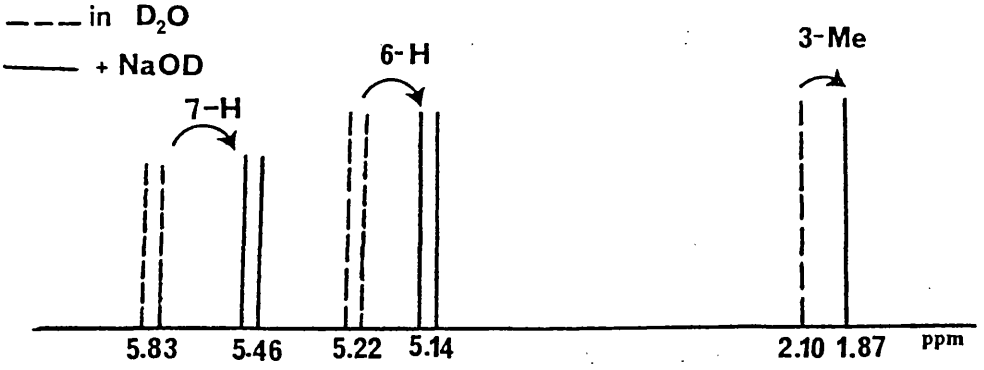
iv) appearance of two signals at 5.55, 5.59 ppm (due to the exo-methylene protons **3**=CH_2 at C-3).

These changes indicate lactam ring cleavage, accompanied by the expulsion of the 3'-substituent and formation of an exo-methylene product.

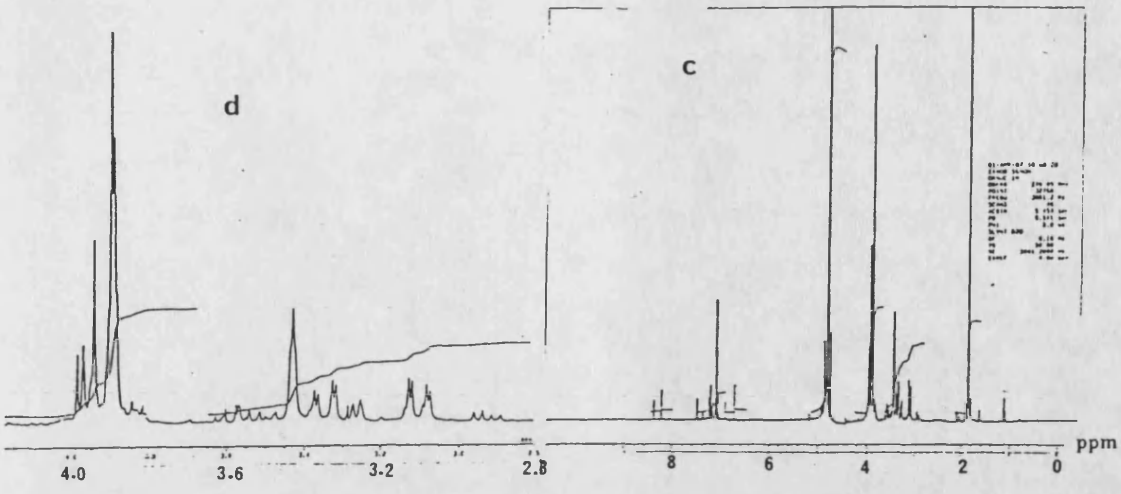
2. Cefotaxime Na

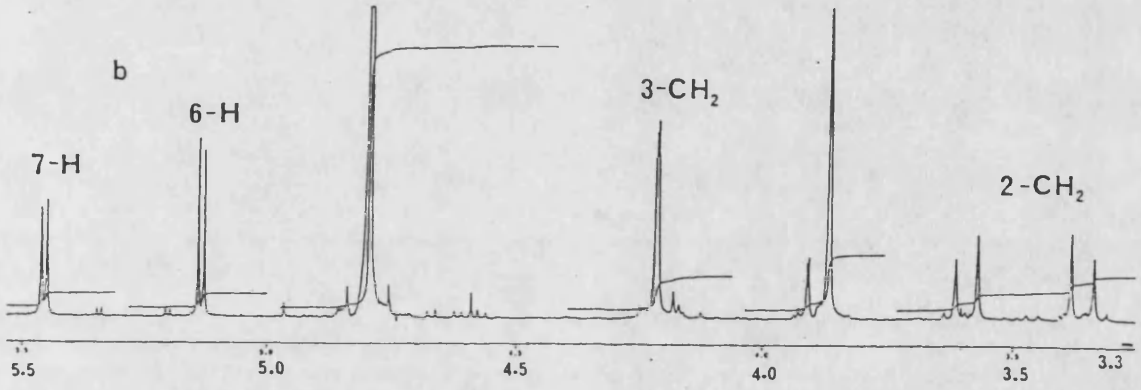


	6-H, 7-H	3'-CH_2	2-CH_2	OCOMe
Intact	5.22,5.83 d	4.72,4.89 d	3.41,3.69 d	2.10 s
	(4.6)	(12.5)	(18.0)	
Hydrolysis	5.14,5.46 d	4.22 dd	3.36,3.59 d	1.87 s
product	(4.7)	(12.0)	(18.0)	



As shown in spectrum (Fig. 4.8, p.159) and the Table above, there is evidence for the β-lactam ring opening only, indicated by the upfield shifts of 6-H/ 7-H signals; no exocyclic methylene signals were observed. The upfield movements of signals due to 3'-CH_2 and OCOMe also indicate deacetylation of the 3'-substituent of the starting compound. It is not possible to decide from the spectrum if the β-lactam ring cleavage and deacetylation is a concerted reaction or otherwise. The spectrum of desacetylcefotaxime in D_2O-$NaHCO_3$ was similar to that of the NaOD-treated solution but different in having 7-H/ 6-H signals at lower field (5.74 and 5.16 ppm respectively).





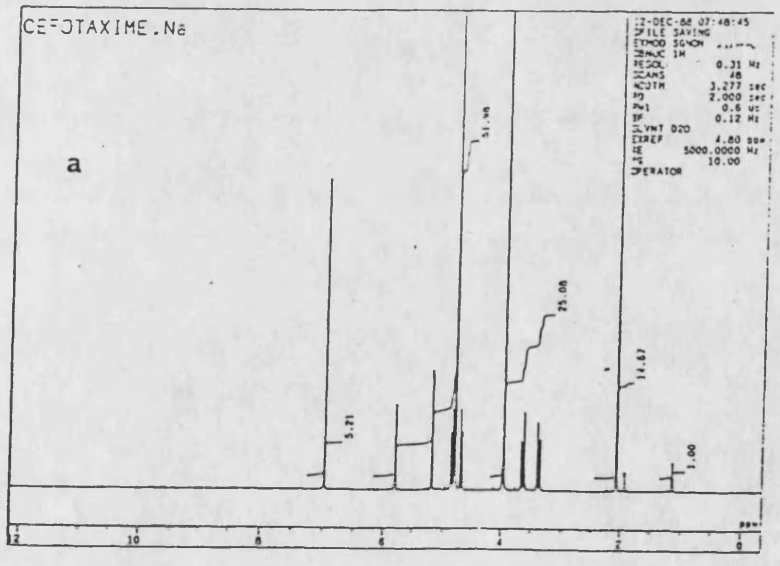
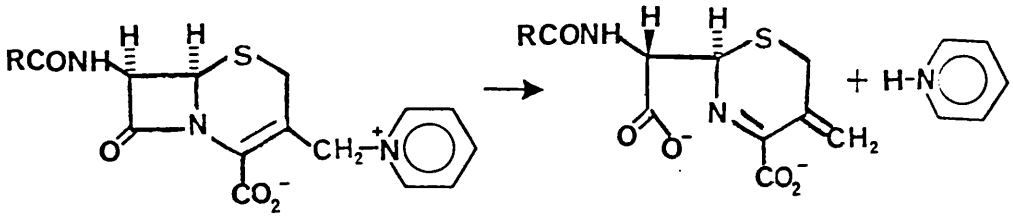
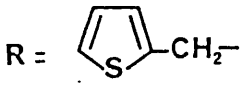


Fig. 4.8 : 270 MHz ^{1}H NMR spectra of cefotaxime Na in a) D_2O, b) immediately after addition of NaOD, and c) two days later. d) Expansion of the 3.4 -5.5 ppm region of spectrum (c).

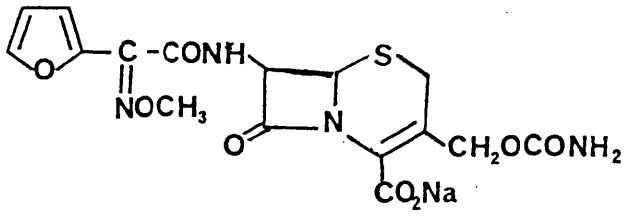
3. Cephaloridine Na

The compound showed rapid β-lactam ring opening (upfield movement of the 6-H/ 7-H signals: 5.07, 5.60 ppm, J=4.8 Hz to 4.50, 5.30 ppm, separation 2.2 Hz) accompanied by removal of the 3'-substituent, a conclusion supported by the disappearance of the double doublet due to 3'-CH_2 at 5.27, 5.48 ppm plus the presence of a neutral pyridine molecule indicated by the upfield shift of its protons (multiplets centred at 8.02, 8.50 and 8.90 ppm moved to 7.30, 7.71 and 8.35 ppm). The formation of the exo-methylene at C-3, was indicated by the pair of lines centred at 5.50 ppm with a separation of ~ 15.0 Hz and integrating for about two protons. Furthermore, it appeared from the small separation (2.2 Hz) of the 6-H, 7-H signals that the hydrolysis product had undergone epimerization at either C-6 or C-7.





4. Cefuroxime Na



A well-resolved spectrum (Fig. 4.9, p.161) was obtained after basification with NaOD. The duplication of signals indicates presence of more than one molecular species in the reaction mixture at the time of recording the spectrum, as shown below:

i) An intermediate with a cleaved β-lactam ring, prior to loss of the carbamic acid anion

from position 3', indicated by the upfield shift of the 6-H, 7-H signals (doublets at 5.23, 5.83 ppm, J= 4.6 Hz moved to 5.12, 5.44 ppm, J=4.9 Hz) and the presence of the double doublet (of reduced intensity), due to 3'-CH_2, shifted upfield (dd at 4.68, 4.87 ppm moved to 4.60 ppm, lowfield doublet obscured by the HDO peak).

ii) An exo-methylene product with cleaved β-lactam ring, indicated by the upfield movements of the 6-H/ 7-H signals (d at 5.12, 5.44 ppm moved to 4.69, 5.38 ppm, separation 3.7 Hz) and appearance of a pair of lines centred 5.58 ppm, integrating for two protons, due to the exo-methylene at C-3.

iii) Epimerization at C-6 and C-7 is another possibilty, indicated by duplication of the 6-H, 7-H, 2-CH_2, NOMe, and the aromatic signals.

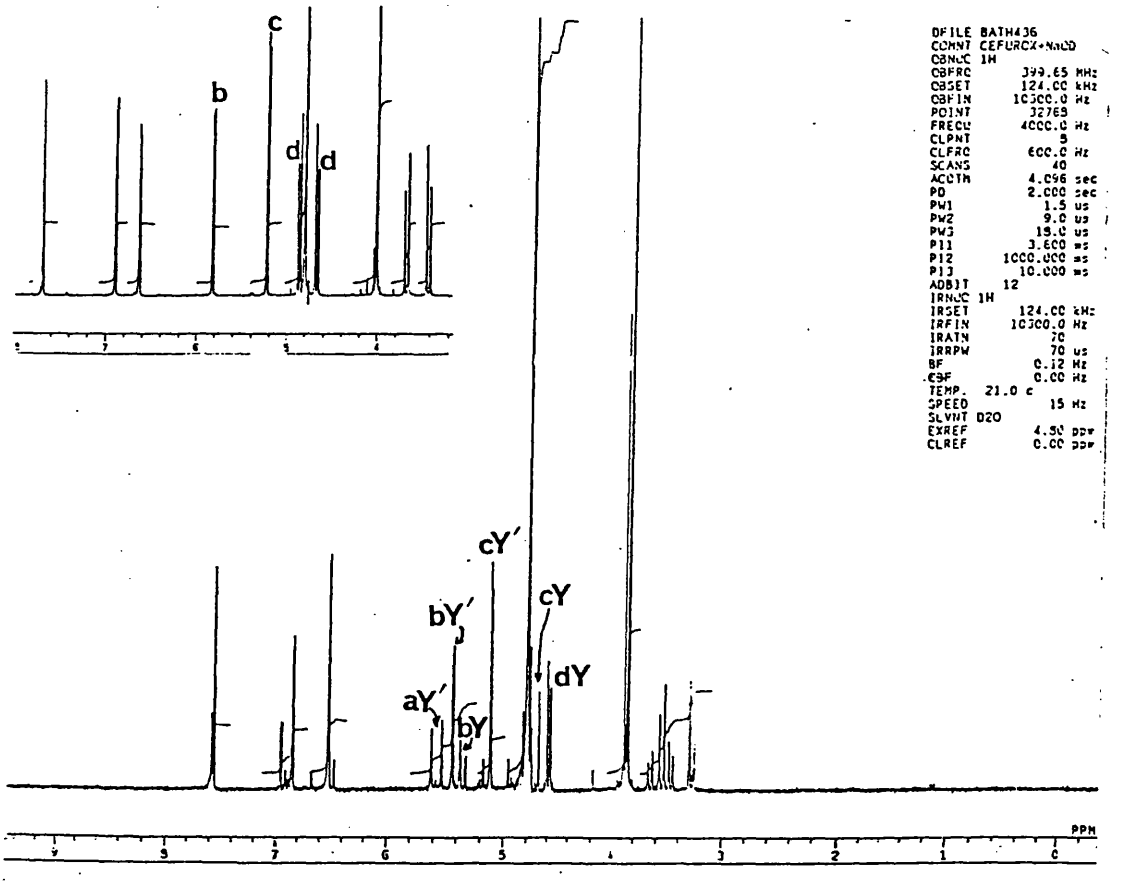
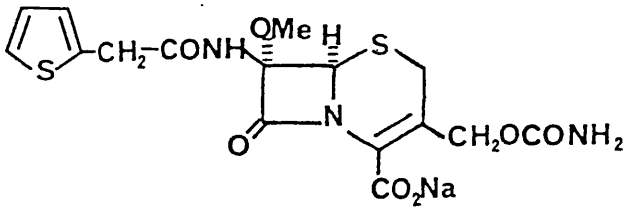


Fig. 4.9 : 270 MHz ^1H NMR spectrum of cefuroxime Na in D_2O + few drops of NaOD.
Insert : normal spectrum in D_2O. a C_3=CH_2 b 7-H c 6-H d C_3--CH_2

Y = intermediate product (with cleaved ring and intact carbamic acid in position-3).

Y'= exo-methylene product (with cleaved ring and less of the carbamic anion from position-3).

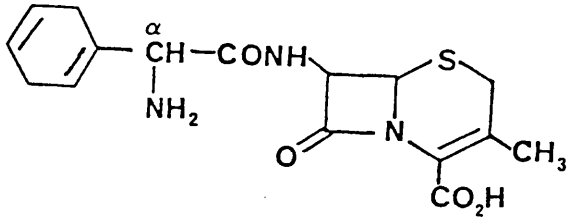
5. Cefoxitin Na



The product obtained from the alkaline hydrolysis showed a complex spectrum, especially the 2.90-3.60 ppm region. Hydrolysis of the lactam ring with expulsion of the 3'-substituent occurred, indicated by the upfield movements of the 6-H and 7-OMe signals and the complete absence of the double doublet due to the 3'-CH_2 of the intact antibiotic, with the formation of the exo-methylene at C-3, indicated by the two signals at 5.45, 5.52 ppm.

b) Cephalosporin antibiotics with no leaving group at C-3

1. Cephradine



	6-H, 7-H	2-CH_2	3-Me
Intact	5.03, 5.57 d (4.4)	2.68, 3.53 d (18.0)	1.86 s
+NaOD		3.20, 3.83 d	1.97 s

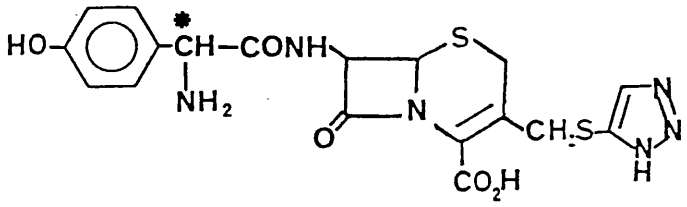
After addition of NaOD, a poor spectrum was obtained, showing cleavage of the β-lactam ring, indicated by the disappearance of the 6-H, 7-H signals. Degradation products, such as a piperazine dione derivative, could not be identified since reference compounds were not available.

2. Cefaclor

Apart from evidence of β-lactam ring opening (absence of 7-H, 6-H signals), the spectrum recorded after addition of NaOD could not be analysed.

c) Miscellaneous cephalosporins

Cefatrizine



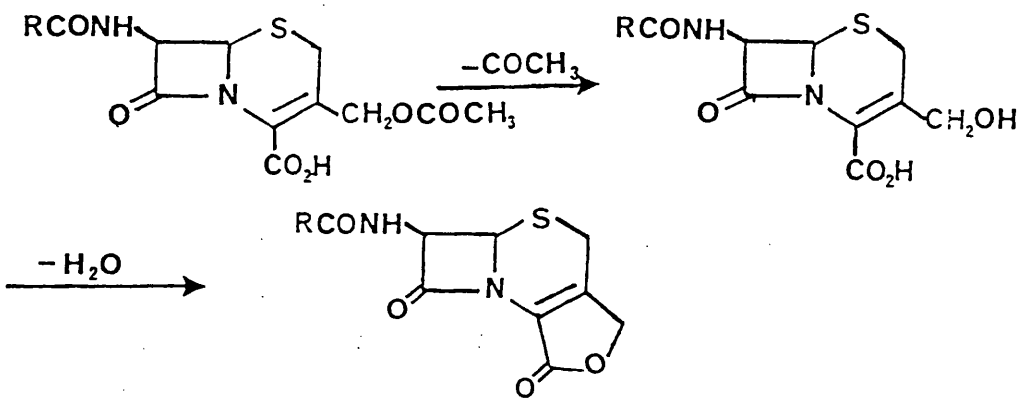
From the spectral changes which occurred after addition of NaOD, it is clear the hydrolysis of the β-lactam ring took place, indicated by the upfield movements of the 6-H, 7-H signals accompanied by a decrease in coupling constant value (4.98, 5.52 ppm, separation 4.4 Hz moved to 4.58, 5.29 ppm, separation 3.2 Hz) together with formation of an exo-methylene signals (as two overlapping doublets at 5.51, 5.52 ppm). Disappearance of the double doublet due to 3'-CH_2 (centred at 3.86 ppm) corresponded to the appearance of the two doublets overlapping at 5.51, 5.52 ppm (J=15.7 Hz). The duplication of signals due to 3'-CH_2(exo), 6-H, 7-H, ArCH and phenolic protons indicated isomerization of the degradation products.

As in all cephalosporins with α-amino substituents in the C-7 amido side chain, diketopiperazine formation is also possible but at this stage no evidence for this route was available.

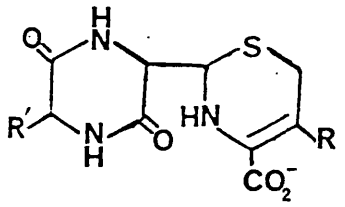
4.4.4 Acid degradation of cephalosporins

4.4.4.1 Introduction

The formation of desacetyl and lactone derivatives as a result of acid hydrolysis of 3-acetoxymethylcephalosporins has already been discussed in section 4.1.2.2, p.120. These cephalosporins may lose their acetyl function to give corresponding desacetyl derivatives which subsequently eliminate water to form the fairly stable lactones (Scheme 4.9, p.120). The lactonization occurs rapidly only at relatively low pH. This route is well documented in the literature[36,75,126–129].



The acidic degradation of the 7α-amino-containing cephalosporins (such as cephalexin and cefaclor) has also been reviewed[67,146] in section 4.1.2.5, p.128. A diketopiperazine-type compound 4.20 was characterised as the major degradation product :



4.20

4.4.4.2 Source of compounds

Sodium salts of cephalothin and cefotaxime were kindly supplied by Eli Lilly and Roussel (U.K), respectively [see Table 2.1, p.25]. Cefotaxime (as free acid) and desacetylcefotaxime were also provided by Roussel. Desacetylcefotaxime sodium and desacetylcefotaxime lactone were obtaied from Hoechst (England).

Δ^3-7-Phenyl–acetamido–desacetoxy–cephalosporin lactone was provided by Professor M.Page, Huddersfield Polytechnic. These compounds were used as received.

The following materials were prepared as described in Chapter two : cephalothin free acid, desacetylcephalothin and desacetylcephalothin lactone

With knowledge of the ^{1}H NMR features which distinguish intact 3-acetoxymethyl cephalosporin antibiotics from their desacetyl and lactone derivatives (see below), experiments were carried out to assess the potential of NMR in monitoring the degradation of cephalosporins of this kind under conditions of low pH.

4.4.4.3 Procedure

(pH < 2 but uncontrolled by a buffer)

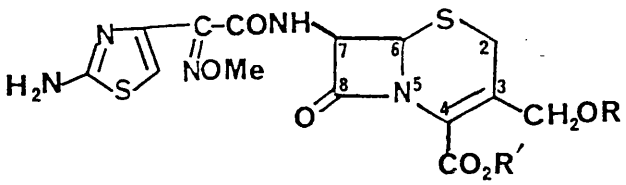
The ^{1}H NMR spectrum of a cephalosporin (mostly Na salt) was run in DMSO-d_6 (D_2O usually added), then re-run after addition of 1-2 drops DCl (pH attained < 2). The spectral changes were followed with time.

Addition of acid will bring about rapid conversion of the sodium salt to the free acid. This may be followed by deacetylation (to 3-CH_2OH) and then lactonization. Deacetylation will produce acetic acid (CH_3COOH) which will have a ^{1}H Me signal that differs from acetylmethyl of the intact antibiotic (generally upfield of antibiotic signal).

DMSO-d_6 was chosen as it is a universal solvent for all likely products.

4.4.4.4 Cefotaxime series (in DMSO-d_6)

Table 4.6 below shows a comparison between the ^{1}H chemical shifts (δ ppm) of the following compounds :



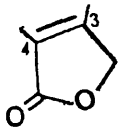
Compound	R	R'
1. Cefotaxime Na	-COMe	Na
2. Cefotaxime free acid	-COMe	H
3. Desacetylcefotaxime	H	H
4. Desacetylcefotaxime lactone		

Table 4.6 : ^{1}H NMR chemical shifts (ppm) of the above compounds 1, 2, 3,and 4 (in DMSO-d_6) :

Compound	7-H	6-H	3-CH_2	NOMe	2-CH_2	OCOMe
1	5.60 dd	5.0 d	4.97,4.73 d	3.84 s	3.47,3.20 d	2.00s
	(4.9,8)	(4.9)	(12.2)		(17.2)	
+D_2O	5.62 d	5.0 d	4.92,4.74 d	3.86 s	3.48,3.23 d	2.02 s
	(4.9)	(4.9)	(12.2)		(17.2)	

2	5.80 dd	5.15 d	4.99,4.68 d	3.84s	3.63,3.49 d	2.04 s
	(4.9,8.2)	(4.9)	(12.8)		(18.2)	
$+D_2O$	4.74 d	5.15 d	4.98,4.73 d	3.84 s	3.63,obscured	2.05 s
	(4.8)	(4.8)				
3	5.56 dd	4.95 d	4.18,nr 3.85dd	3.84 s	3.47,3.29dd	
	(4.7,8)	(4.6)	(12-13)		(18.0)	
$+D_2O$	5.63 d	5.0 d	4.23,4.03 dd	3.89 s	3.50,3.37 dd	
	(4.6)	(4.6)	(12.8)		(17.4)	
4	5.94 dd	5.16 d	5.05 brs	3.85 s	3.79 dd	
	(4.9,8.2)	(5.2)				
$+D_2O$	5.9 d	5.18 d	5.04 brs	3.88 s	3.83,3.73 dd	
	(4.9)	(4.9)			(18.8)	

Footnotes : s=singlet, brs=broad singlet, d=doublet, dd=double doublet nr=near, J values (Hz) in parenthesis.
$+D_2O$= few drops of D_2O added to the cephalosporin solution in DMSO-d_6.

Notes from Table 4.6 :

i) The 7-H, 6-H, 3-CH_2 and 2-CH_2 signals of desacetylcefotaxime 3 are at higher fields compared to those of free acid 2 (most pronounced for 3-CH_2 and valuable as an aid to differentiation).

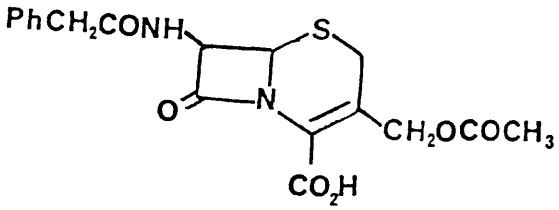
ii) Cefotaxime lactone 4 is differentiated from cefotaxime free acid and desacetyl 3 by the broad singlet nature of 3-CH_2 (exo); also 2-CH_2 is lower field of free acid and desacetyl signals with smaller AB chemical shift differences.

iii) The signals due to 7-NH and Ar-NH_2 (>7.00 ppm) are absent after D_2O exchange; in addition, the 7-H signal changes from a double doublet to a doublet.

iv) In the lactone 4 the 2-CH_2 and 3-CH_2 protons are almost equivalent appearing as broad

singlets or narrow multiplets. This is presumably a result of the molecule being more planar than the parent antibiotic whereby differences between the environments of CH_2 pairs are small.

The lactone derived from the cephalosporin 4.38 showed similar characteristics



4.38

to that of cefotaxime lactone. The ^1H NMR spectral (in DMSO-d_6 + D_2O) data were as follows : 7-H/ 6-H 5.84, 5.10 ppm (d, 5.0 Hz), 3-CH_2 5.05 ppm brs, 2-CH_2 3.83, 3.74 ppm (d, 18.6 Hz) [3.79 ppm as brs in absence of D_2O], ArCH_2 3.59, 3.51ppm (d, 14.1 Hz).

The chemical shift data of Table 4.6, measured from spectra of solutions in DMSO-d_6 at near-neutral pH may differ in solutions of lower pH (as in proposed experiments). This was checked by recording spectra in DMSO-d_6 plus DCl; the spectral data (in ppm) were as follows :

Cefotaxime Lactone :

7-H 5.9 d, 6-H 5.2 d, 3-CH_2 5.07 brs, NOMe 4.02 s, 2-CH_2 3.89, 3.79 d (separation 18.6 Hz). i.e. little change apart from small chemical shift differences.

Desacetyl derivative :

Acidification of this derivative would be expected to lead to lactonization :

a) Freshly acidified solution : 7-H, 6-H minor doublet pair at 5.90, 5.23 ppm, major doublet pair at 5.74, 5.16 ppm.

b) Two hours later : 7-H, 6-H 5.9/ 5.23 ppm and 5.74/ 5.16 ppm doublets were of similar intensities.

It may be concluded that the 5.9/ 5.23 ppm doublet pair is due to the lactone and the 5.74/ 5.16 ppm pair to the desacetyl derivative in the free acid state, as from Table 4.6 :

7-H/6-H 5.63/5.00 ppm.

c) 7 days later : the spectrum was essentially similar to that of the pure lactone :

7-H, 6-H 5.90, 5.24 ppm doublets,

3'-CH_2 5.07 ppm broad singlet,

2-CH_2 3.83, 3.51 ppm doublets.

2-CH_2 :

Both fresh and 2 hour aged spectra showed a pair of 4-line signals which could be assigned to 2-CH_2 of the lactone (3.89, 3.77ppm, d) and desacetyl derivative (3.68, 3.57 ppm, d), compared to 2-CH_2 of desacetyl cefotaxime (3.50, 3.37 ppm, d) and cefotaxime lactone (3.83, 3.73 ppm, d) of Table 4.6 (in DMSO-d_6+D_2O).

Spectra of cefotaxime Na run in DMSO-d_6 + DCl :

a) Freshly acidified solution (Fig. 4.10a, p.170) :

The high intensity of OCOMe signal at 2.11 ppm showed that most of the antibiotic was intact at this stage. A lower intensity signal at 2.07 ppm was evidence of some loss of acetyl. 7-H/ 6-H, 3-CH_2 and 2-CH_2 signals of the intact molecule were also prominent. Minor signals of the lactone were resolved : 7-H 5.92 ppm (overlapped HDO band), 6-H 5.30 ppm , 3-CH_2 at 5.12 ppm (as broad singlet), 2-CH_2 as double doublet centred near 3.87 ppm. No signals due to the desacetyl intermediate were detected.

b) After 60 minutes :

Signals due to intact antibiotic and lactone were approximately of equal intensities. The upfield shift of the OCOMe signal (from 2.11 ppm to 2.07 ppm) is a characteristic of acetate. The broad singlet at 5.12 ppm, integrating for two protons, was that of the methylene protons

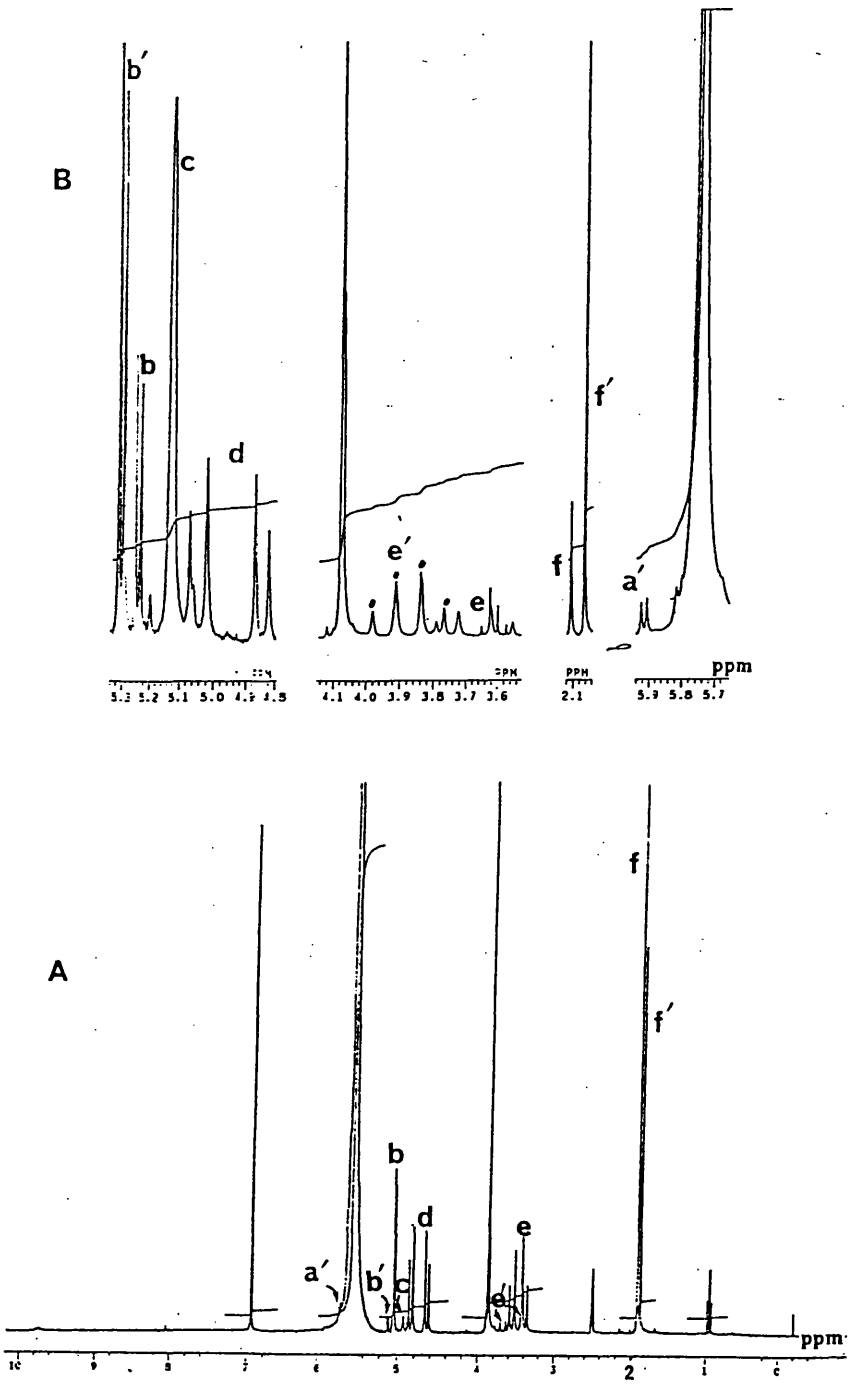


Fig. 4.10 : Partial ^{1}H NMR spectra of cefotaxime Na in DMSO-d_6 + DCl : A) recorded immediately after acidification. Note scale error (add 0.2 ppm), and B) run 2 hrs later.

a: CT 7-H b: CT 6-H c: lactone 3'-CH_2

a': lactone 7-H b': lactone 6-H d: CT 3'-CH_2

e : CT 2-CH_2 f : CT 3'-Me

e': lactone 2-CH_2 f': Me of acetate group

CT = Cefotaxime

of the lactone ring. The chemical shift difference of the 2-CH_2 of lactone are smaller (0.14 ppm) than those of the intact antibiotic (0.17 ppm)

c) Two hours after acidification (Fig. 4.10b, p.170) :

The lactone signals were of greater intensity than those of the intact molecule (~ 2:1).

d) 40 hours later :

The spectrum was essentially of lactone signals only. The acetate Me signal appeared as a singlet at 2.07 ppm. Those of the lactone molecule were : 2-CH_2 3.94, 3.80 ppm (double doublet, J=18.7 Hz), NOMe 4.07 ppm (singlet, 3H), lactone CH_2 at 5.12 ppm (broad singlet, 2H), 6-H at 5.30 ppm (doublet, J=5.0 Hz), 7-H 5.92 ppm (doublet, J=5.0 Hz) and ArH at 7.14 ppm (singlet, 1H).

In a second trial (Fig. 4.11, p.172), a spectrum of cefotaxime Na in DMSO-d_6, D_2O + DCl showed that the lactone preponderated in the freshly run solution. The acetate Me signal appeared as an intense singlet at 1.95 ppm, while lactone signals were as follows : double doublet centred at 3.85 ppm due to 2-CH_2, lactone CH_2 at 5.07 ppm as broad singlet, 6-H 5.22 ppm (doublet, J=4.9 Hz), and 7-H at 5.91 ppm (doublet, J=4.9 Hz). It was specially fortunate that the HDO band did not obscure the signals in the spectrum.

The above acid products signals showed similar spectral characteristics to that of authentic lactone sample run in DMSO-d_6 plus DCl : 7-H 5.9 ppm (doublet), 6-H 5.2 ppm (doublet), 3-CH_2 5.07 (broad singlet), NOMe 4.02 ppm (singlet), 2-CH_2 3.89, 3.79 ppm (double doublet, J=18.6 Hz).

A spectrum run 5 days later displayed lactone signals only.

In no case could the presence of the desacetyl intermediate be detected.

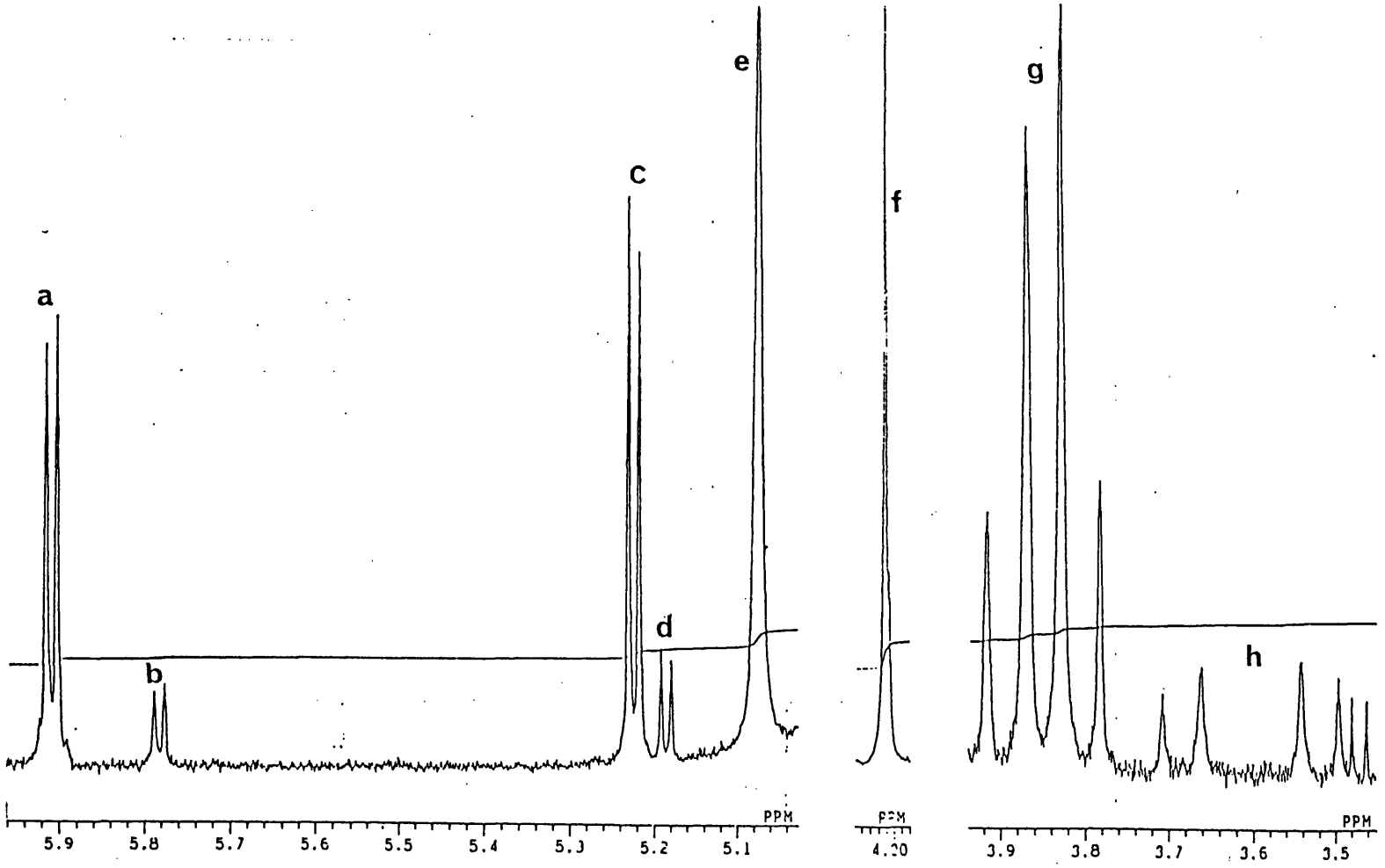


Fig.4.11 : Partial (expanded) ^{1}H NMR spectrum of cefotaxime Na in DMSO-d_6, D_2O + DCl showing the following signals :

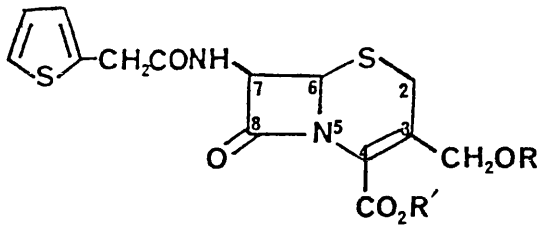
a) lactone 7-H c) lactone 6-H e) lactone 3'-CH_2 (CT 3'-CH_2 obscured by HDO band)

b) CT 7-H d) CT 6-H f) NOMe g) lactone 2-CH_2 h) CT 2-CH_2

CT = Cefotaxime

4.4.4.5 Cephalothin series (in DMSO-d_6)

Table 4.7 below shows a comparison between the ^1H chemical shifts (δ ppm) of the following compounds :



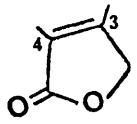
Compound	R	R'
1. Cephalothin Na	OCMe	Na
2. Cephalothin free acid	OCMe	H
3. Desacetylcephalthin	H	H
4. Desacetylcephalothin lactone		

Table 4.7 : ^1H chemical shifts (in ppm) of 1, 2, 3 and 4[a] (in DMSO-d_6)

Compound	7-H	6-H	3-CH_2	2-CH_2	OCOMe
1	5.50 d	4.94 d	4.97, 4.75 d	3.47, 3.22 d	2.01 s
	(4.6)	(4.6)	(12.1)	(17.2)	
2	5.70 dd	5.10 d	5.01, 4.68 d	3.63, 3.49 d	2.03 s
3	5.48 dd	4.90 d	4.18, 3.86 d	3.48, 3.31 d	
	(4.9,8.4)	(4.9)	(12.4)	(17.7)	
4	5.90 dd	5.20 d	5.17 brs	3.49 s	
	(5.1,8.4)	(5.1)			

a For abbreviations see footnotes under Table 4.6.

Relationships amongst this series are similar to those found for the cefotaxime group. Again the 3-CH_2 and 2-CH_2 resonances of the lactone show the effects of a reduced CH_2 chemical shift difference in each case.

Spectra of cephalothin Na run in DMSO-d_6-DCl

a) Freshly run

The spectrum was essentially that of the free acid. The intensity of the acetylmethyl signal at 2.05 ppm gave evidence of the small degree of degradation at this stage. The rest of the antibiotic signals had high intensities and minor resonances were not resolved. These signals were : 7-H at 5.70 (double doublet, 4.8 Hz), 6-H 5.10 ppm (doublet, 4.8 Hz), 3-CH_2 5.01, 4.68 ppm (as double doublet, 12.8 Hz), 2-CH_2 3.63, 3.49 ppm (double doublet. 18.2 Hz). OCOMe as singlet 2.03 ppm. No signals due to desacetylcephalothin (as intermediate) were observed.

b) 6 days later

The 7-H 5.82 ppm (doublet, 5.0 Hz), 6-H 5.13 ppm (doublet, 5.0 Hz), 3-CH_2 5.07 ppm as broad singlet, 2-CH_2 double doublet centred 3.82 ppm signals of the lactone molecule were more intense than those of the intact antibiotic. A single methyl signal at 1.94 ppm (due to acetate) was present. Minor 7-H, 6-H and OCOMe signals of the antibiotic were resolved.

c) 30 days later : Only lactone signals were present.

Some experiments were carried out to detect de-acetylation of cefotaxime and cephalothin without subsequent lactonization by use of a pD 4.4 buffer in 1:1 DMSO-d_6/ D_2O (by dissolving 30 mg of KH_2PO_4 and 50 mg of Na_2HPO_4 in sufficient D_2O to produce 10 ml and adjusting the pH to 4.0 with glacial acetic acid).

The spectrum of cefotaxime free acid run immediately after dissolution in this buffer was that of a single product. Chemical shifts of signals due to 7-H (d, 5.71 ppm), 6-H (d, 5.1 ppm) and 2-CH_2 (3.60, 3.35 ppm d) were close to those of both the intact antibiotic and its desacetyl derivative (Table 4.6, p.166) with no evidence of the presence of lactone. However the presence of a four line signal centred near 4.81 ppm due to the 3-CH_2 protons and

absence of a signal of this kind near 4.0 ppm gave evidence that loss of the acetyl group had not occurred.

This conclusion was confirmed by re-running the spectrum after the addition of authentic desactylcefotaxime when clear duplication of the 7-H and 6-H doublets was seen together with the appearance of a new 3-CH_2 signal centred near 4.2 ppm.

A similar result was obtained when a solution of cephalothin Na in the pD 4.0 buffer was examined. The gap between deacetylation and lactonization of 3-acetoxy methyl cephalosporins must therefore be a narrow one.

4.4.4.6 Notes on acid-degradation of other cephalosporins (in DMSO-d_6 + DCl) :

1. Cephaloridine Na

Only small changes were observed after addition of DCl. These involved the slight low field shift of the 7-H (from 5.56 ppm to 5.75 ppm), 6-H (5.05 ppm to 5.20 ppm), 3-CH_2 (the double doublet centred at 3.50, 3.10 ppm moved to 3.60, 3.40 ppm). The pyridyl signals showed very little difference after acidification (9.15, 8.60, 8.15 ppm to 9.05, 8.66, 8.22 ppm) as further evidence of the molecule still being intact. The above low field shifts could be explained as the change of the molecule to that of the free acid. If cephaloridine eliminated pyridine (protonated) in the presence of acid, it should yield a lactone identical to that formed from cephalothin, No evidence of lactone formation was obtained.

2. Ceftazidime Na

It behaved similarly to cephaloridine. No signals due to the lactone were detected even 5 days after addition of DCl.

3. Cefsulodin Na

No change other than slight lowfield shift of signals. The molecule remained intact.

4.4.4.7 ^{13}C NMR diagnostic features of lactones

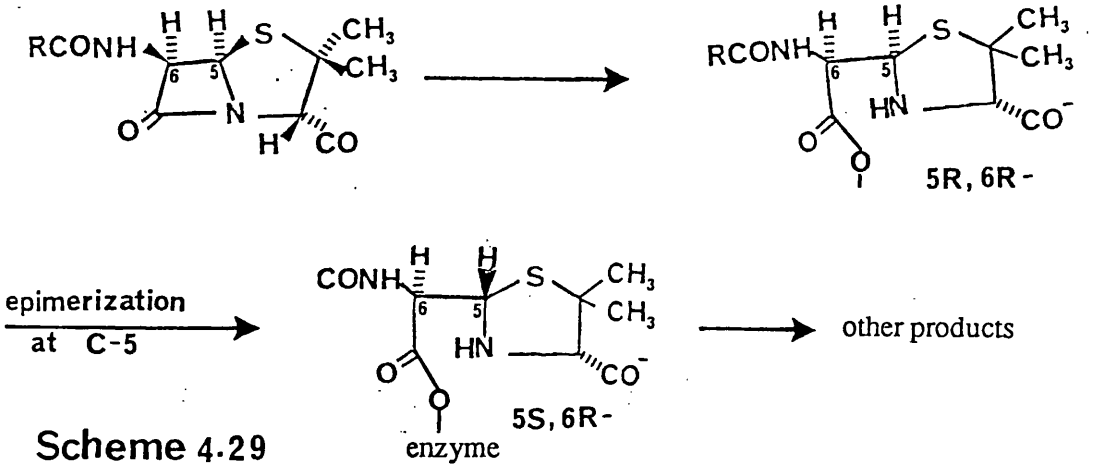
The ^{13}C NMR spectra of lactones derived from cefotaxime, cephalothin and the 7-benzylamido derivative 4.38 (p.168) were recorded in DMSO-d_6. Spectra were similar to those of the parent cephalosporins but could be distinguished by the 3-CH_2 resonance. The 3-CH_2 resonance moves to lower field when the lactone ring forms, as shown below :

3-CH_2	free acid (ppm)	lactone (ppm)
Cefotaxime	63.8	72.0
Cephalothin (Na in D_2O)	64.7	71.5
7-benzylamido derivative 4.38	unknown	71.3

Supplement (Chapter 4)

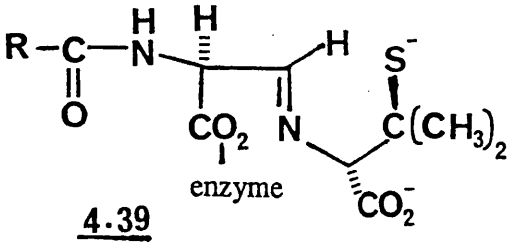
" Investigation of ability of ^1H NMR to monitor the degradation of penicillin antibiotics by β-lactamase enzymes (see p.135). "

Treatment of a penicillin antibiotic with a β-lactamase enzyme would be expected to open the β-lactam ring to give the corresponding 5R, 6R penicilloic acid. On storage the acid of 5R, 6R configuration should then isomerize to the 5S, 6R diastereoisomer and possibly to other isomers and further products of degradation (Scheme 4.29 below).



Scheme 4.29

Epimerization is known to proceed at C-5 (not C-6) because deuterium exchange does not occur at C-6 when the reaction is performed in D_2O, a fact which supports interconversion via the imine tautomer 4.39[121].



4.39

The reactions of Scheme 4.29 may be readily monitored by ^1H NMR by recording spectra of benzylpenicillin in D_2O (Fig.4.12a) treated with a small amount of NaOD/ D_2O. Spectra are shown in Figure 4.12, p.179.

a) 5 min after addition of alkali

No intact antibiotic remains (absence of 5-H, 6-H signals) and the spectrum is that of the 5R, 6R penicilloic acid.

b) 60 min after start (Fig.4.12b)

Low intensity signals due to 5-H and 2-Me_2 of the 5S, 6R penicilloic acid are now apparent. These exceeded the intensities of corresponding 5R, 6R signals at 5 hrs (Fig.4.12c), and at 24 hrs. After 24 hrs SR signals predominated by a factor of about 8.

Some NMR experiments, chiefly qualitative, of the effect of β-lactamase enzymes on several penicillin antibiotics were next carried out.

The procedure was to dissolve an arbitrary amount of a sample of β-lactamase (obtained from Genzyme Biochemicals Ltd) in D_2O and to add a small volume (equal in all cases) to a solution of the antibiotic (20-30 mg in 0.5 ml D_2O). The enzyme and antibiotic solutions were stored at 5°C when not in use. Viability of the enzyme solution was assessed by determining its action on the NMR spectra of benzylpenicillin.

In the first experiment, a spectrum of benzylpenicillin sodium in D_2O was recorded. A few drops of a solution of enzyme obtained from Genzyme were added to the solution in the NMR tube and spectra recorded at intervals. No buffer system was present.

The 5 minute spectrum (Fig.4.13a, p.180) shows that a mixture of intact antibiotic and 5R, 6R benzylpenicilloic acid are present. Unexpectedly the chemical shifts of the penicilloic acid (notably 5-H, 6-H and 3-H) were distinctly lower field than corresponding signals observed for the acid generated in D_2O-NaOD [Table 4.8, p.181]. This must be a pH effect. The acid will exist as a dianion at high pH (D_2O-NaOD) whence the carboxylate functions exert maximum shielding influences on nearby protons (Scheme 4.30, p.181).

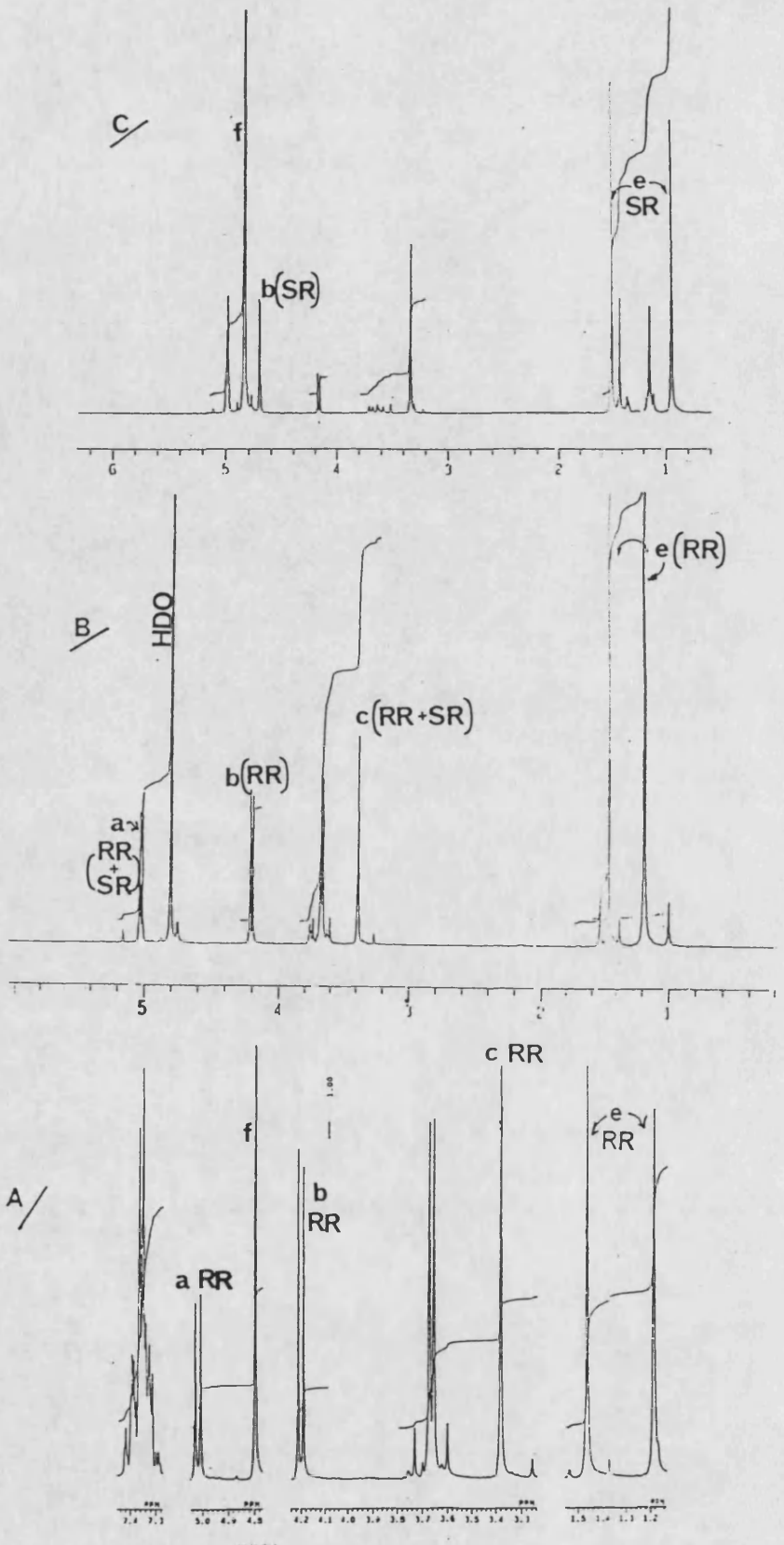


Fig.4.12 : 270 MHz ^{1}H NMR spectra of benzylpenicillin Na in D_2O A) 5 min after addition of NaOD, B) 60 min after start of experiment, and C) 5 hrs later.

RR = 5R, 6R-benzylpenicilloic acid SR = 5S, 6R-benzylpenicilloic acid.

a 6-H b 5-H c 3-H d $PhCH_2$ e 2-Me_2 f HDO

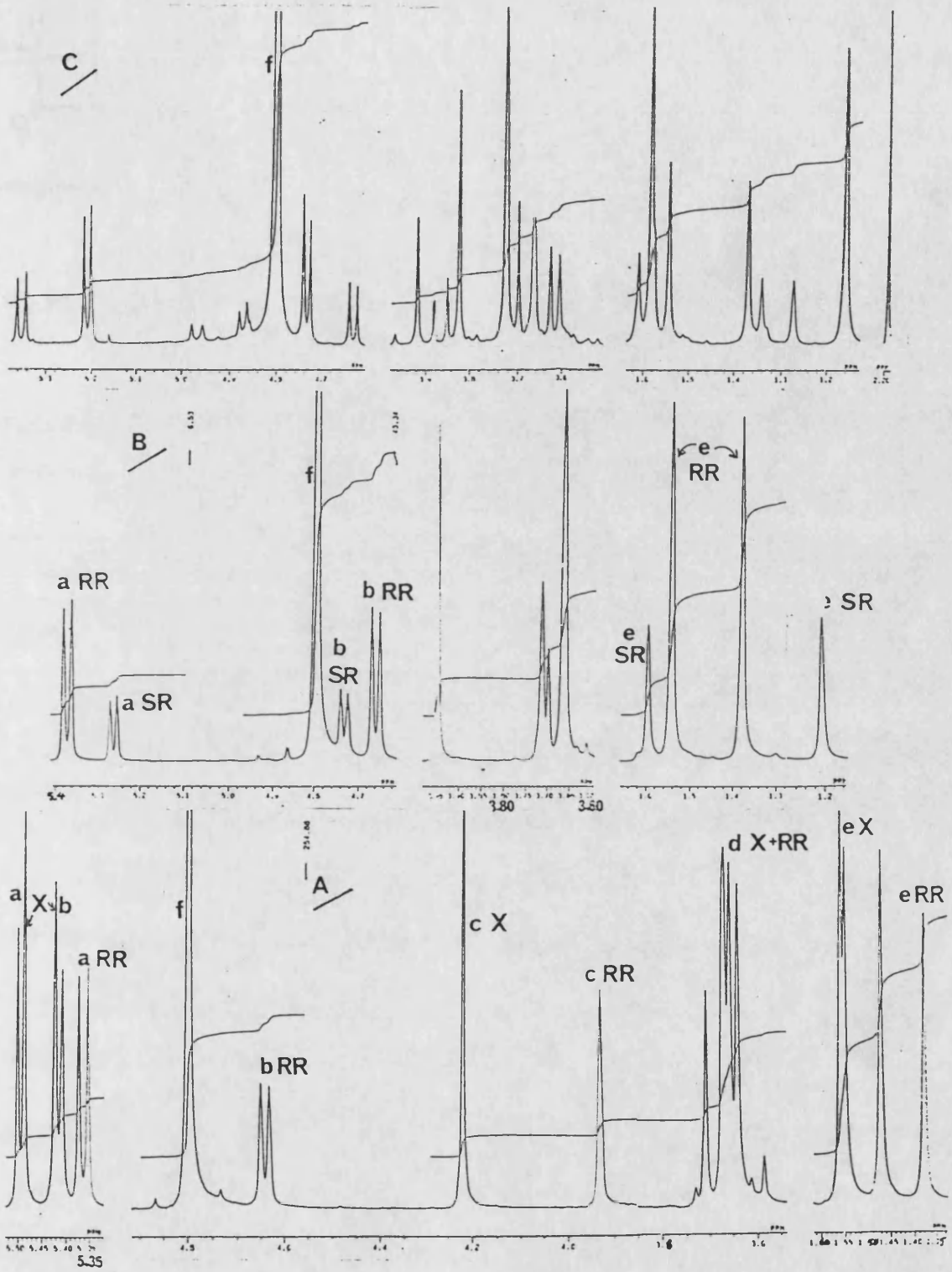
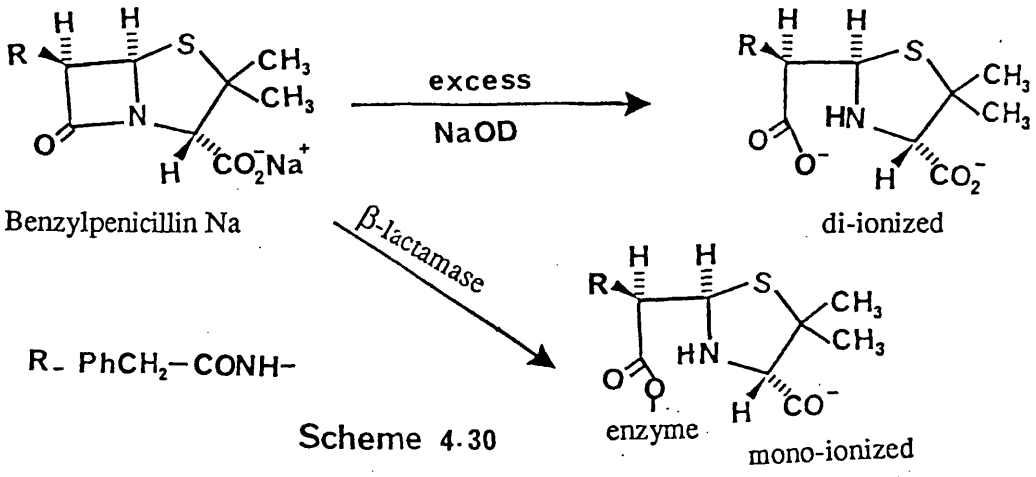


Fig.4.13 : ^{1}H NMR spectra of benzylpenicillin Na + β-lactamase (Genzyme) : A) recorded 5 min after addition of enzyme, B) 9 hrs after start of experiment, and C) 4 days later. (same annotations as for Fig.4.12). See text p.178, 181 for details.

X = Intact antibiotic.



Scheme 4.30

Table 4.8 : Some of the ^{1}H NMR data (in ppm) of benzylpenicillin (intact) and its immediate enzymatic and alkaline products

Compound	6-H	5-H	3-H
Intact	5.5 d	5.43 d	4.22 s
	(3.85 Hz)	(3.84)	
Enzymatic	5.36 d	4.64 d	3.93s
product	(4.95)	(4.95)	
Alkaline	5.01 d	4.20 d	3.38 s
product(+NaOD)	(6.0)	(6.0)	

d=doublet, s=singlet, J values in Hz in parenthesis.

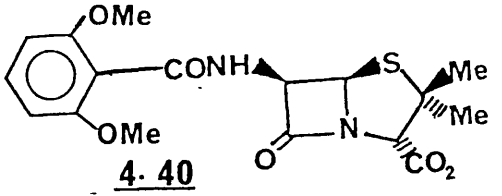
After 45 minutes the spectrum was principally that of the 5R, 6R penicilloic acid; all penicillin signals had disappeared.

After 9 hrs, the spectrum was that of a mixture of 5R, 6R and 5S, 6R epimers with excess of the former (Fig.4.13b).

In the 24 hr spectrum the epimers ratio was approximately 1:1.

After 4 days, the 5S, 6R diastereoisomer was the major component of the mixture. Signals due to a third product were now apparent (see narrow doublets near 5 ppm and the 2-Me_2 region which shows 7-8 lines instead of the 4 indicative of a binary mixture, Fig.4.13c, p.180).

Methicillin Na was the next example to be studied.



4.40

The spectrum of material (provided by Beecham laboratories) that had been stored at 5°C for several years was consistent with the β-lactam structure 4.40 but showed low intensity doublets (near 5.03 and 5.28 ppm) and singlets (1.49 and 1.66 ppm) indicative of the presence of a small amount of one of the diastereoisomeric penicilloic acids.

After addition of the Genzyme enzyme little spectral change was seen after 5 hrs (Fig.4.14 below) while even after 24 hrs signals due to the intact antibiotic remained predominant.

These results concur with the proven resistance of methicillin to penicillinases[155].

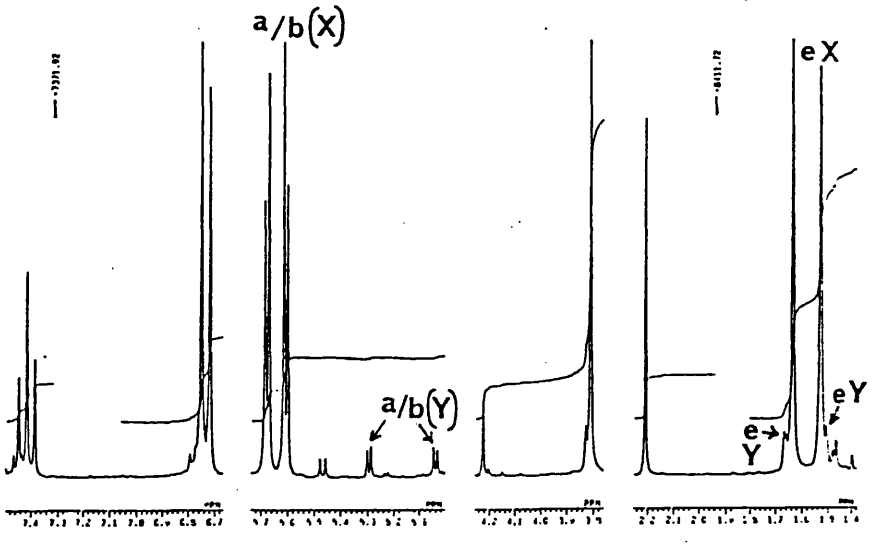
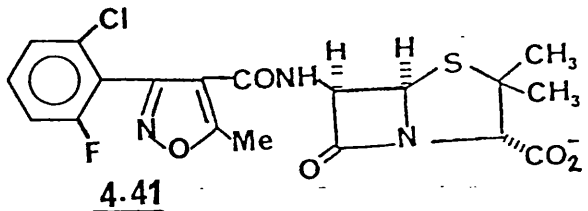


Fig.4.14 : 270 MHz ^{1}H NMR spectrum of 4.40 5 hrs after addition of β-lactamase from Genzyme, showing the stability of the antibiotic to the enzyme.
X=intact antibiotic signal, Y=penicilloic acid signals.
(For abbreviations see annotations under Fig.4.12, p.179).

Flucloxacillin Na



4.41

The ^{1}H NMR spectrum of a sample of flucloxacillin Na (4.41) in D_2O indicated that the sample was pure (5-H, 6-H doublets 5.5, 5.6 ppm, 2-Me_2 singlets 1.45 and 1.49 ppm). After addition of the Genzyme enzyme little change was seen after 10 minutes but a 90 minute spectrum (Fig.4.15, p.183) showed lower intensity doublets near 4.7 and 5.3 ppm and two new lines in the 2-Me_2 region (1.36 and 1.57 ppm). After 29 hrs, signals due to the intact antibiotic remained the major resonances and there was some evidence of epimerization of the initially formed penicilloic acid (new doublets near HDO resonance, two new lines in the 2-Me_2 region).

Results of an experiment using oxacillin Na were similar. Both these isoxazole derivatives are of proven resistance to penicillinases[155].

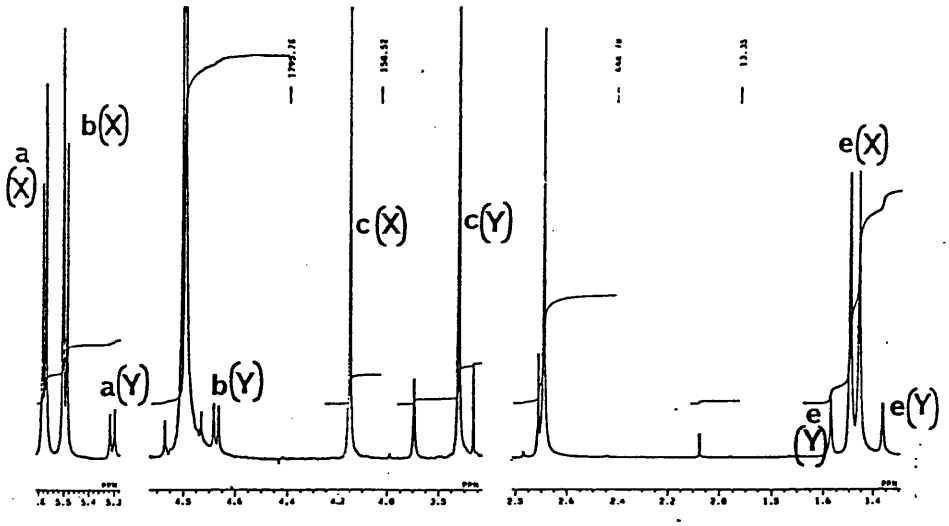
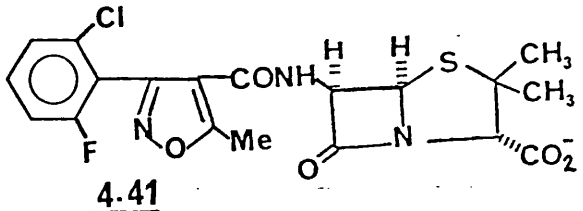


Fig.4.15 : 270 MHz ^{1}H NMR spectrum of flucloxacillin Na + Genzyme enzyme 90 min after commencement of experiment.

X= intact antibiotic signals, Y=penicilloic acid signals.

(For abbreviations see annotations under Fig.4.12, p.179).

Flucloxacillin Na



4.41

The ^{1}H NMR spectrum of a sample of flucloxacillin Na (4.41) in D_2O indicated that the sample was pure (5-H, 6-H doublets 5.5, 5.6 ppm, 2-Me_2 singlets 1.45 and 1.49 ppm). After addition of the Genzyme enzyme little change was seen after 10 minutes but a 90 minute spectrum (Fig.4.15, p.183) showed lower intensity doublets near 4.7 and 5.3 ppm and two new lines in the 2-Me_2 region (1.36 and 1.57 ppm). After 29 hrs, signals due to the intact antibiotic remained the major resonances and there was some evidence of epimerization of the initially formed penicilloic acid (new doublets near HDO resonance, two new lines in the 2-Me_2 region).

Results of an experiment using oxacillin Na were similar. Both these isoxazole derivatives are of proven resistance to penicillinases[155].

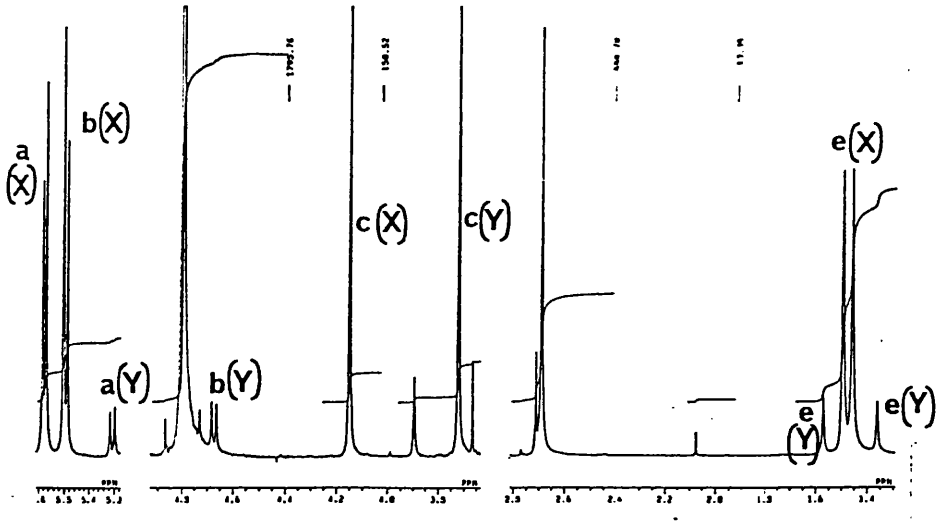
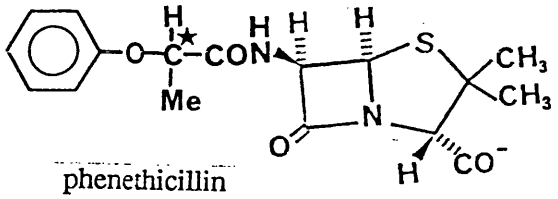


Fig.4.15 : 270 MHz ^{1}H NMR spectrum of flucloxacillin Na + Genzyme enzyme 90 min after commencement of experiment.

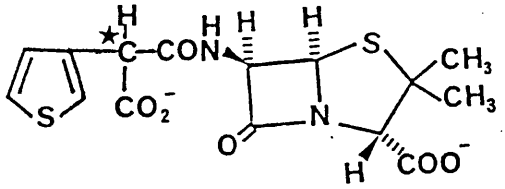
X= intact antibiotic signals, Y=penicilloic acid signals.

(For abbreviations see annotations under Fig.4.12, p.179).

The next two examples of phenethicillin and ticarcillin present additional complexity in that they are marketed as diastereoisomeric mixtures epimeric about the α-carbon of the 6-amido side chain :



phenethicillin



ticarcillin

Phenethicillin K

The 270 MHz ^{1}H NMR spectrum of the sample showed 2 sets of 5-H/ 6-H doublets in the 5.3-5.6 ppm region and 4 lines in the 2-Me_2 region in addition to the duplication of other resonances (Fig.4.16a, p.185). A spectrum recorded 15 min after addition of the Genzyme enzyme (Fig.4.16b) displayed the same features but changed in the following respects :

1) the highest field doublet of the 5-H/ 6-H signal (in the 5.4-5.3 ppm region) was more complex showing overlap with one or more additional signals.

2) a new pair of doublets were present 4.65 ppm.

3) the 2-Me_2 region now showed two new lines (at 1.34 and 1.39 ppm).

A spectrum recorded two hours after the start of the experiment showed the following features :

1) 5-H/ 6-H signals due to the intact antibiotic were absent while those due to the principle penicilloic acids were well resolved near 5.4 and 4.65 ppm. The low intensity doublets near 5.2 and 5.3 ppm are probable due to the corresponding epimers.

2) In the 2-Me_2 region. lines due to the intact molecule were absent while four lines of the principle penicilloic acids were prominent plus low intensity lines due to epimers.

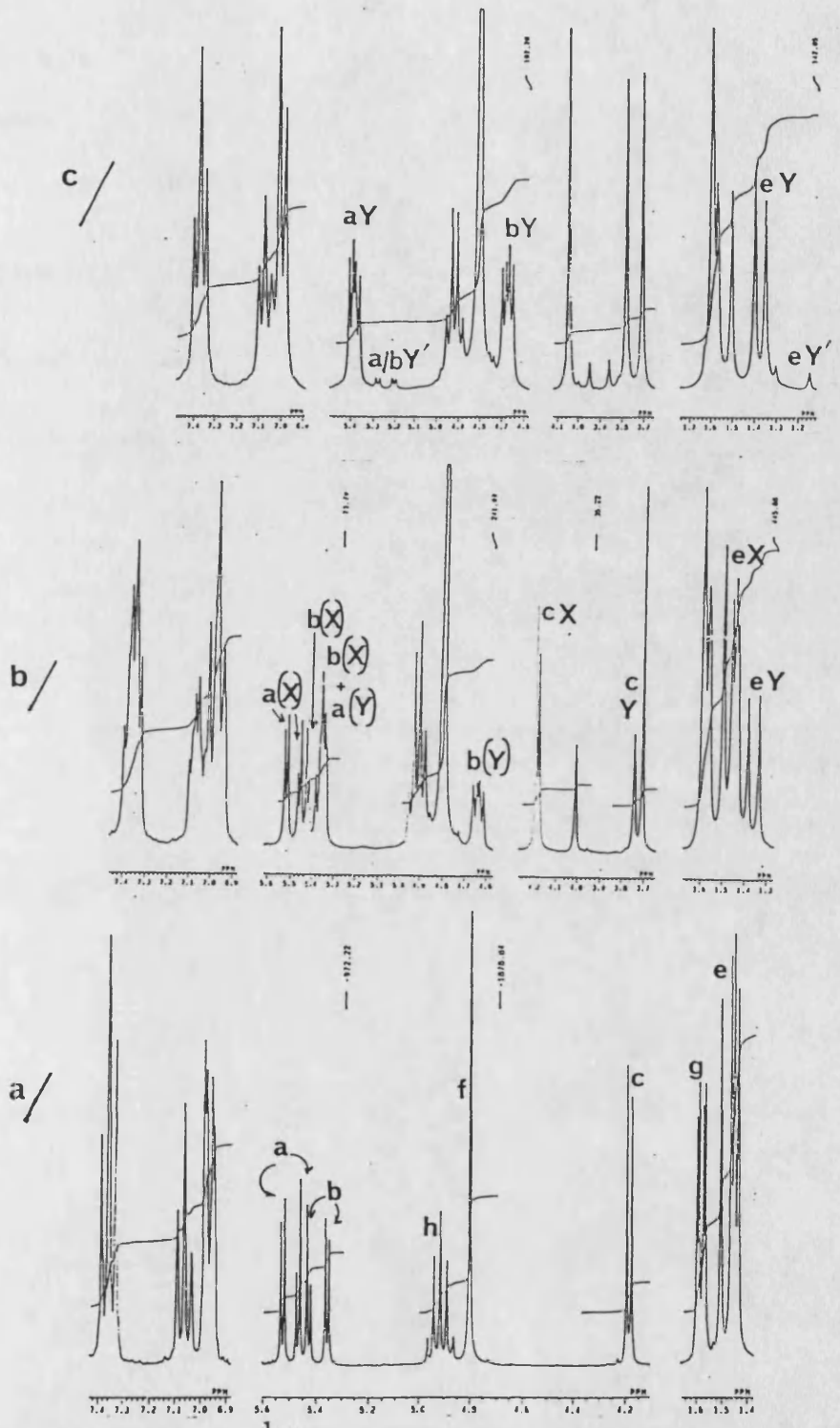


Fig.4.16 : The 270 MHz ^{1}H NMR spectra of phenecillin Na a) in D_2O, b) 15 min after addition of Genzyme enzyme and c) recorded 2 hrs after the start of the experiment.

X=intact diastereoisomers, Y=5R,6R penicilloic acids Y'=5S,6R penicilloic acids

g CHMe h PhOCH. (For other abbreviations see annotations under Fig.4.12, p.179).

A 24 hrs spectrum showed the same features but intensities of signals due to epimers had increased relative to the original penicilloic acids.

Under approximated equivalent conditions it is clear that phenethicillin is far more susceptible to the Genzyme enzyme than methicillin, flucloxacillin and oxacillin.

Ticarcillin Na

The spectrum of the sample in D_2O showed a complex signal in the 5-H/ 6-H region analysed into a high field doublet and a low field pair of doublets the inner lines of which overlapped the doublet which corresponded with that at high field. The 2-Me_2 region displayed three lines, the central being made up of two closely overlapping lines (Fig.4.17a, p.187). These features confirm the diastereoisomeric nature of the material.

Spectral changes apparent 5 min after addition of the Genzyme enzyme were as follows:

1) appearance of an apparent triplet (overlapping doublets) centred near 5.3 ppm, assigned to ring opened products.

2) presence of four extra lines in the 2-Me_2 region (Fig.4.17b).

A 24 hr spectrum (Fig.4.17c) had the following features :

1) 5-H/ 6-H signals due to intact antibiotic were absent.

2) 5-H/ 6-H signals due to the initial penicillioc acids and their epimers were well resolved (overlapping doublets near 5.36 ppm and 5.25 ppm, 5-lines to high field of HDO band near 4.8 ppm).

3) eight lines in the 2-Me_2 region none of which corresponded with those of the intact antibiotic.

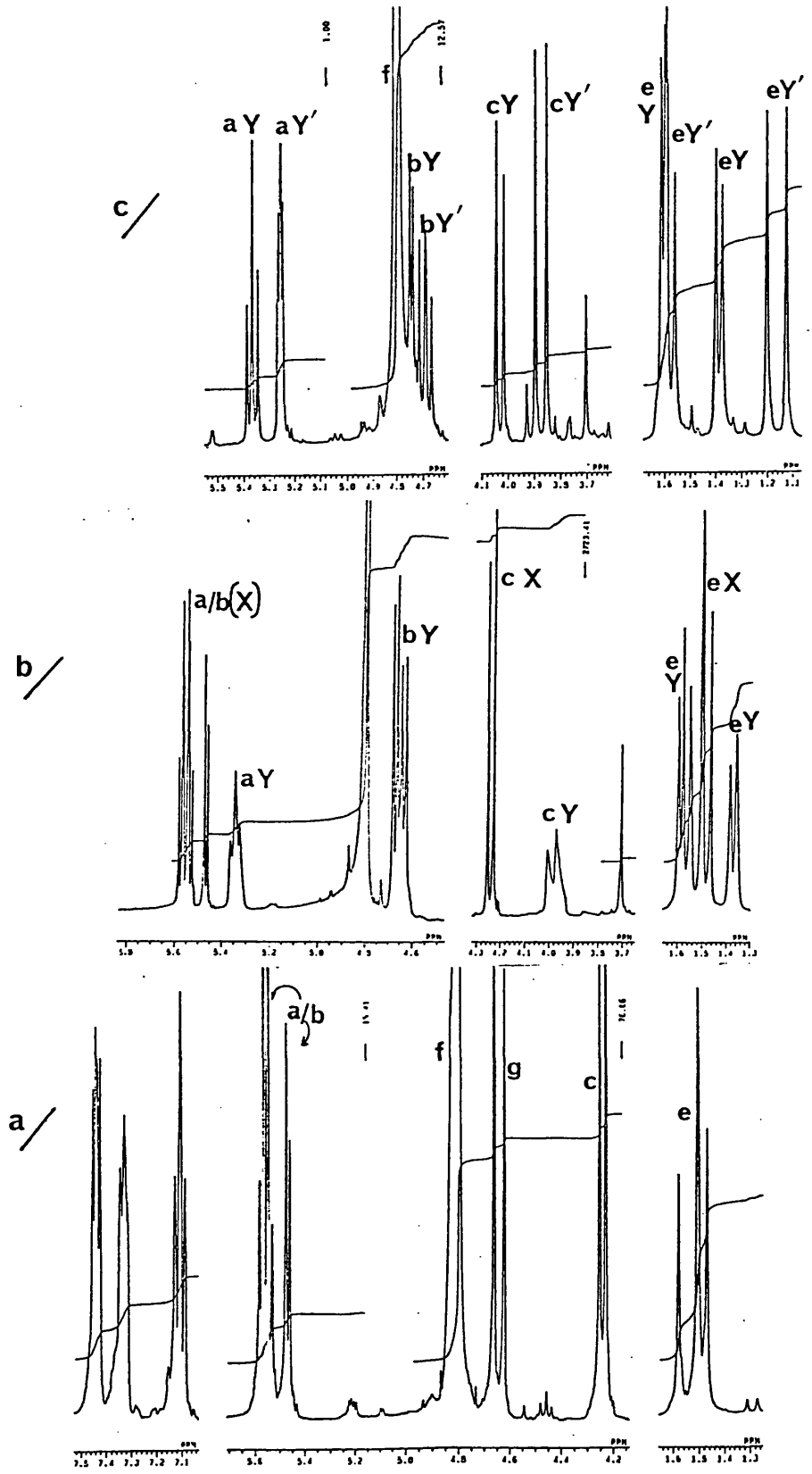


Fig.4.17 : The 270 MHz ^{1}H NMR spectra of ticarcillin Na a) in D_2O, b) 5 min after addition of Genzyme enzyme, and c) 24 hrs after start of experiment.

X=intact diastereoisomers, Y=5R,6R penicilloic acids Y'=5S,6R penicilloic acids

g ArCH. (For other abbreviations see annotations under Fig.4.12, p.179).

Spectra run at the time intervals of 60 min and 8 hrs although poorly resolved, clearly indicated the absence of 5-H/ 6-H signals due to the intact antibiotic.

Susceptibility of ticarcillin to the Genzyme enzyme is demonstrated by these results together with evidence of epimerization of the initially produced β-lactam ring-opened products.

CHAPTER FIVE

Investigation of binding of cephalosporins and degradation products to serum albumins

5.1 Introduction

The binding of drugs to plasma proteins is of remarkable biological significance, as it influences the distribution of drugs in the body and their access to sites of action, metabolism and excretion[157,158]. Drug distribution is represented schematically in Figure 5.1 below. Therefore, an understanding of the nature of this binding is clearly fundamental to the prediction of the therapeutic and toxic effects of drugs.

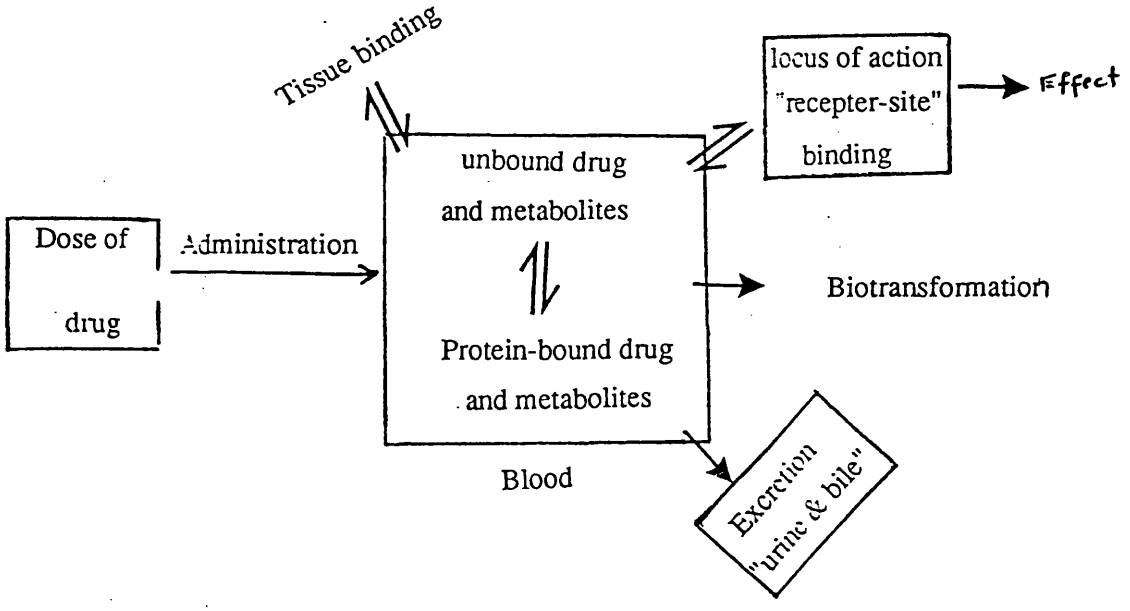


Fig.5.1 : Schematic diagram of effect of protein binding on drug pharmacokinetics.

Drug-protein interactions cover a wide range of binding mechanisms, from the highly specific binding of a drug to a 'receptor-site', for instance, physostigmine inhibition of acetylcholinesterase action, to the relatively non-specific interaction between various compounds and the plasma proteins[159]. This specificity of binding at receptor sites is logical since an enzyme needs only to have an action on the substrate it is designed for, and a pharmacological receptor would be of reduced value if a wide variety of substances caused

an effect. Apart from tendency to favour lipid-soluble molecules, plasma protein binding is remarkably non-specific. It occurs with drugs that are aromatic (such as suphonamides) or heterocyclic (streptomycin), anionic (penicillins) or cationic (phenothiazines), water-soluble (sodium salicylate) or lipid-soluble (barbiturates), and with those that have polar (amino acids) or neutral (bilirubin) groups. Almost all drugs are bound reversibly to proteins throughout the body. The amount of drug bound is a function of the concentration of the drug, the affinity of the drug for the binding sites, and the capacity of the binding sites[160,161].

When drug molecules are bound to plasma proteins, they are pharmacologically inert. However, binding of drugs to serum proteins in vivo has major clinical significance when levels of free drugs are reduced to values below the effective concentrations[160,162]. Furthermore, bound drug molecules can not gain access to the sites of biotransformation or excretion, and because protein binding is reversible, the bound drug serves as a reservoir which releases unbound drug when needed. As it is generally accepted that only free, unbound drug can exert pharmacological activity[162], the binding of drugs to serum proteins can have a considerable effect on their potency and duration of action. One example of these effects is the highly protein bound trypanicidal drug Suramin which remains in the body for a considerable time, but enough is released from the bound form to exert a trypanicidal action for 3 months or more after administration of a single intravenous dose[160,163].

A number of endogenous substances, such as fatty acids, bilirubin, hormones etc., are bound to plasma proteins, competing for the same limited number of protein sites as for drugs, and therefore affecting the binding of these drugs. Also the ability of one drug to displace another depends on the competition for common binding sites. A compound which is strongly bound will displace a weakly bound one. This can have a marked pharmacological effect. The action of some drugs may be mediated, increased. or decreased by other drugs or endogenous compounds by displacement from protein binding sites. In some cases, such a

displacement could be advantageous, but in other cases it may be dangerous[163]. For instance, a reduction of only 0.6% in serum binding of dicloxacillin by sulphaethylthiadiazole increases the concentration of unbound dicloxacillin at its locus of action by 20-40%[164]. Cases have been reported in which phenylbutazone has caused profound bleeding in patients under treatment with the anticoagulent drug warfarin[165]. Also, displacement of bilirubin from plasma albumin in newborn infants by acidic drugs such as salicylates, causes Kernicterus (brain damage)[163].

The most important contribution to drug-plasma protein binding is made by serum albumin which comprises about half of the total plasma proteins[157,158], and because it interacts with a wider variety of drugs, due to its comparatively large surface area[159], than do other plasma proteins, it is used frequently in model investigations of drug binding[157]. The albumin molecule consists of a single polypeptide chain with a molecular weight of about 69,000. At the plasma pH (7.4), it has a net anionic charge (the isoelectric point being at pH 4.9)[166]. Albumin has high capacity (i.e., several binding sites of one type), but low affinity for binding most cationic drugs. However, many acidic drugs bind strongly to albumin; many of them are bound with high affinity but the binding sites may have only a low capacity (<10)[167], and in most cases not more than two primary sites[168]. Many drugs also bind to lower affinity (secondary) binding sites, which often number less than ten.

It has been suggested that there are potentially only three distinct sites for most drugs on human albumin[158,169,170]. These have been characterised by the use of fluorescent probes[169] and are designated sites I, II, and III. Site I is also referred to as the warfarin-binding site, site II as the indole and benzodiazepine binding site, and site III as the digitoxin site.

The binding affinity depends strongly upon the molecular structure of the drug. It appears that the terminal and side chain carboxyl and hydroxyl groups of the amino acid residues of the albumin, which are predominantly ionized at the pH of plasma, are likely to

be involved in the binding of drugs[157]. It is generally considered that albumin can attract drugs by forces of association between ionic charges, polar and non-polar groups, through the establishment of, ionic, charge transfer, hydrophobic, van der Waals or hydrogen bond linkages[171]. More than one of these forces can be involved in a single combination. In most cases the binding force seems to be principally electrostatic, involving the squeezing out of the intervening water layers, as both the drug and protein are surrounded wholly or partly by water molecules. This interaction as a whole is sometimes described as hydrophobic bonding, involving conformational changes in the drug and the albumin molecules[157–159,172].

5.2 β-Lactam antibiotics and plasma protein binding

As the majority of infections occur in the tissue rather than the vascular system, the protein binding of antimicrobials is of considerable importance in the assessment of their efficacy. A relationship between the proportion of drug bound and the penetration into tissues has been studied[173]. Generally, it appeared that a highly bound antibiotic (>80%) penetrated poorly into the tissue. Although there is a controversy over the relevance of protein binding in antibiotic therapy[174], it appears that there is to some extent a linear relationship between penetration of free drugs into tissues and protein binding[160,173].

The cephalosporins form a chemically and pharmacologically homogeneous group, but they are known to bind with different affinities, i.e. strong, medium and weak binding, to serum proteins[175]. The consequences of this binding on bacteriological activity, rate of renal excretion, and body distribution of these drugs have received considerable attention[176,177]. For example, Barza et al.[176] have shown that the serum concentration of the β-lactam antibiotics are proportional to the rate constant of elimination of the drug, and the volume of distribution. Only free antibiotic manifests antibacterial activity; e.g., cephalothin in serum in a concentration of 100 μg/ ml exhibits an antibacterial effect similar to that of 10 μg/ ml in water. Thus a high degree of serum binding may nullify the apparent advantages of higher

serum levels of some agents. Another important consequence of protein binding is related to the fact that only free drug is able to cross the "aqueous pores" of the capillary endothelium[199].

The binding of cephalosporins by human and bovine serum albumin has been investigated by several authors with different techniques. It is evident from Table 5.1 (p.194), which summarises the literature data, that the results reported show considerable variation. A general review of studies on the binding of cephalosporins to plasma proteins has been published by Nightingale[177], who reported the binding expressed as a %. This data is presented in Table 5.2, below. A more detailed description of the techniques used to measure binding in the present and other work follows in section 5.2.

Table 5.2 : Binding data of some cephalosporins for HSA as obtained from Nightingale review[177]

Compound	Technique	Binding data (in %)
Cephalexin	Miscellaneous	< 10
Cephradine	Miscellaneous	8 - 30
Cephazolin	Equilibrium dialysis	73 - 84
	Ultrafiltration	74 - 86
	Others	80 - 90
Cephalothin	Ultrafiltration	65
Cephaloridine	Miscellaneous	8 - 31

Briand and coworkers[175] examined the binding of seven cephalosporins to human serum albumin (HSA) with equilibrium dialysis and proton NMR. From their results, Table 5.1, cephalexin does not bind to HSA, and the binding of cephradine is very weak (K_a ~ 300 M^{-1}). Cephapirin, cefoxitin, and cefotaxime have only moderate affinity for HSA (K_a around 1000-2000 M^{-1}). Strong binding was seen for cephamandole and cephazolin (K_a greater than 5000 M^{-1}). These values showed some agreement with previously published

Table 5.1 : Binding data for cephalosporins as obtained by previous investigators using different techniques :

Cpd[a]	blood fraction	Technique	Binding data n, K_a (M^{-1})	Reference
CEP		Ultrafiltration	56 %	177
		Ultrafiltration	65 %	162
	BSA	Fluorescence probe	2.2, 1.2 x 10^3	180
	BSA	Fluorescence probe	3.0, 7.0 x 10^3	185
	HSA	Fluorescence probe	2.0, 0.85 x 10^3	180
	HSA	Fluorescence probe	3.0, 3.3 x 10^3	186
CEZ		Ultrafiltration	74 %	162
	HSA	Equilibrium dialysis	0.7, 2000	175
	BSA	Fluorescence probe	3.0, 8.19 x 10^3	185
	HSA	Fluorescence probe	3.0, 6.36 x 10^3	186
	HSA	Fluorescence probe	1.4, 3.67 x 10^5	179
CEM	HSA	Equilibrium dialysis	2.2, 910	175
	BSA	Fluorescence probe	3.0, 8.98 x 10^3	185
	HSA	Fluorescence probe	3.0, 6.6 x 10^3	186
	HSA	Fluorescence probe	1.4, 8.11 x 10^5	179
CED		Ultrafiltration	31 %	177
		Ultrafiltration	20 %	162
	BSA	Fluorescence probe	2.2, 0.59 x 10^3	180
	BSA	Fluorescence probe	3.0, 5.3 x 10^3	185
	HSA	Fluorescence probe	2.0, 0.5 x 10^3	180
	HSA	Fluorescence probe	3.0, 2.86 x 10^3	186
	HSA	Fluorescence probe	1.4, 8.11 x 10^5	179
CEL		Ultrafiltration	15 %	162
	HSA	Equilibrium dialysis	0.0, <100	175
	BSA	Fluorescence probe	2.2, 0.4 x 10^3	180
	HSA	Fluorescence probe	2.0, 0.4 x 10^3	180
	HSA	Fluorescence probe	1.4, 8.57 x 10^5	179
CET	HSA	Equilibrium dialysis	3.3, 710	175
	HSA	Fluorescence probe	1.4, 4.81 x 10^5	179
CER	BSA	Fluorescence probe	3.0, 7.98 x 10^3	185
	HSA	Fluorescence probe	3.0, 4.37 x 10^3	186
CEF	HSA	Equilibrium dialysis	2.0, 6000	175
	HSA	Fluorescence probe	1.4, 6.68 x 10^5	179

a cpd=compound, CEP=cephalothin, CEZ=cephazolin, CEM=cefotaxime, CED=cephaloridine, CEL=cephalexin, CET=cefoxitin, CER=cefuroxime, CEF=cefamandole.

results[174,176–178]. Five of these cephalosporins were examined by Csiba (1985)[179], using the fluorescence probe technique. According to the latter, cefotaxime and cephamandole were bound more weakly to albumin.

Craig and Welling[162] evaluated the serum-protein binding of cephalosporins according to other authors. The examined methods were based on ultrafiltration and equilibrium dialysis. The binding of four cephalosporins in order of rank were, cephalexin 15%, cephaloridine 20%, cephalothin 65%, and cephazolin 86%. The orders were not identical to those obtained by Csiba.

Veronese and coworkers[180] utilised the fluorescent probe technique to determine the binding data of three cephalosporins for serum albumins from different species, namely, human, bovine, rabbit and chicken. Only minor variations in the binding properties were found. The constants for fluorescent probe, ANS, are in agreement with those reported by Jun et al.[181], but disagree with those of Csiba (see Table 5.1). According to Veronese, the number of binding sites in the protein molecule is about two for bovine and human serum albumin, which is also similar to the values reported by Chignell (1969)[182], Sudlow et al. (1973)[183], and Muller and Wolbert (1976)[184]. The association constants for cephalosporins fell into different groups, cephalothin showed a stronger binding affinity (1.0×10^3 M^{-1}), whereas weaker bindings were observed for cephaloridine and cephalexin ($K_a = 0.55 \times 10^3$ and 0.4×10^3 M^{-1}, respectively). These data are in good agreement with those reported by Nightingale[177]; but showed wide variations with those reported by Csiba[179], who observed that weaker binding does take place with cephalothin (Table 5.1).

Kim et al.[185] determined, by a fluorescent probe technique, the binding affinity of six cephalosporins to bovine serum albumin (BSA). In another report[186], they obtained the binding values of the same cephalosporins to HSA, using the same technique. They demonstrated close similarity in binding values of the cephalosporins to BSA and HSA, i.e., stronger bindings for cefotaxime and cephazolin, and weaker bindings for cephaloridine and

cephacetrile (see Table 5.1, p.194). The number of binding sites are similar for both BSA and HSA (n=3).

The pioneering NMR studies by Jardetzky and coworkers[187–189] exemplify the advantages of NMR methods in studying the interactions involved in intermolecular complexes. In particular, proton relaxation rates (spin-spin, $1/T_2$, and spin-lattice, $1/T_1$) provide information about the portion of the ligand which interacts with the macromolecule.

Our literature search revealed only a few NMR studies of the interactions between serum albumins and β-lactam antibiotics. Fischer et al.[189] utilised the transverse (spin-spin) relaxation measurements technique to examine the binding of benzylpenicillin to bovine serum albumin. They demonstrated that there were changes in relaxation rates large enough to be measured. They were able to show that the phenyl group plays the major role in the binding. Briand et al.[175] studied the interaction between human serum albumin and some cephalosporins (cephradine, cephamandole, cefoxitin, and cefotaxime) by using the transverse relaxation rate, $1/T_2$, technique. They postulated that the major binding site for cephalosporins with high HSA affinity (cephamandole and cephazolin) is the electron-rich heterocycle fixed on the methylene at position 3. In those with moderate affinity (cefoxitin and cefotaxime) the binding to the protein involves the heterocyclic substituent of the acetamide chain carbon atom (C-7).

5.3 Techniques used to measure drug-protein binding

Various experimental techniques have been employed to study drug-protein interactions, mainly to elucidate the extent of binding[190,191]. These are listed in Table 5.3 (p.198), with brief notes on their advantages and disadvantages. Chignell(1972)[191] has reviewed a number of these techniques. Further information can be derived from the references given in the Table. All the available methods utilise one of four basic principles (Goldstein, 1949)[192] :

(i) The concentration of free drug may be diminished in the presence of a binding protein.

(ii) The drug may show measurable changes in its properties, on binding.

(iii) Measurable alteration in the protein's properties may occur on drug binding.

(iv) The drug's interaction may be studied by its ability to displace a 'probe compound' from protein-binding site(s).

The most commonly used techniques for studying cephalosporin-protein binding include ultrafiltration, equilibrium dialysis, and fluorescence spectroscopy. With respect to the two former techniques, usually the protein-bound amount of the drug cannot be measured directly but is calculated from the difference between the unbound and the total amount. Ultrafiltration involves the use of special membranes. These are filled with albumin solutions containing the cephalosporin and centrifuged for a period of time. The unbound drug concentration in the filtrate is then determined (e.g., using gas chromatography). The binding parameters of the drug bound to albumin can then be obtained by the Scatchard plot (section 5.3.1). This method was utilised by Craig and Welling[162] to study the binding of four cephalosporins (cephalexin, cephaloridine, cephalothin, and cephazolin) to serum albumin,(Table 5.1, p.194). The main disadvantage of this technique is the possible loss of drug due to adsorption to the membrane.[193] Equilibrium dialysis involves the use of an isotonic phosphate buffer. Drug solutions in the buffer and plasma samples were placed on each side of equilibrium dialysis cells. At equilibrium the concentration of the drug in the buffer solution equals the concentration of the free amount in the protein-drug solution[194]. This technique was used by Briand et al.[175] to examine six cephalosporins, cephazolin, cephamandole, cefotaxime, cefoxitin, cephalexin, and cephradine, (Table 5.1). Although no loss of drug occurs in this technique compared to the previous, but prolonged equilibration time may cause protein denaturation, in addition to the effect of the buffer in disturbing equilibrium by dilution.

Displacement methods employing fluorescence spectroscopy (one of the techniques used in the present work) have proved to be of increasing value and more popular than the other

Table 5.3 Principal methods of studying Drug-Protein Binding[158]

Technique	Advantages	Disadvantages	Selected references
Equilibrium dialysis	Accurate reproducible quantitative data. Most used technique.	Quantitative information only, subject to error from binding to membrane. Prolonged equilibration time causing bacterial contamination and protein denaturation.	210
Ultrafiltration	Rapid. Closely approximate in vivo situation.	Quantitative data only. Binding to membrane. Equilibration disturbed by protein concentration.	211
Ultracentrifugation	No membrane binding.	Prolonged experimental time. Quantitation more difficult. Cannot use protein mixtures.	212
Gel chromatography	Separation of several binding proteins possible	Adsorption to gel may disturb equilibrium. Dilution occurs.	213
Electrophoresis	Qualitative data. Can separate multi-protein mixtures and only small samples required	Quantification difficult. Non-physiological. Interpretation difficult due to binding to support medium.	214
Fluorescence spectrophotometry	Sensitive technique. Can monitor fluorescence changes in both drug/protein on binding. Provides both quantitative and qualitative information. Only micro-quantities of material required.	Can not be used if interaction causes no spectral change. Non-physiological. Need to compensate for compound's absorbance. Quantification can involve lengthy calculation.	215
NMR	Sensitive to changes in molecular geometry	Interpretation may be difficult. Equipment expensive. Non physiological.	188

techniques in protein-binding studies. They involve the ability of some drugs to displace model fluorescent probe molecules from protein-binding sites. Therefore, it can give information on specific-site interactions[158].

The chemical interaction of cephalosporins with serum albumins was also examined by a few workers using NMR resonance methods, i.e., relaxation time (T_1 & T_2) measurements. These techniques provide information on structural features of the studied drug which contribute to the formation of a complex with protein. It seems difficult to judge the validity of results obtained by the relaxation measurements, partly because of the difficulty of making accurate and reproducible measurements of relaxation times since many factors contribute to them[163].

The transverse (spin-spin, T_2) relaxation technique is briefly defined as the redistrbution of the absorbed energy within the spin system to return to equilibrium[195]. It involves the measurement of changes in the linewidths at half heights of the resonance signals arising from the various hydrogen-containing parts of the drug molecules on adding serum albumin. The part involved in protein-drug binding shows relatively larger broadening of its signals. Briand and coworkers[175] utilised this technique in the identification of the drug sites involved in cephalosprin-protein interaction. They calculated the transverse relaxation rate values from the expression, $1/T_2 = \pi\Delta\nu_{1/2}$, where $\Delta\nu_{1/2}$, the full-linewidth at half-maximal height, is the mean of several spectra recorded at a 90° excitation pulse and an acquisition time of 2.6 s.

The longitudinal (spin-lattice, T_1) relaxation process involves the loss of energy from the excited nuclear spins to the surrounding molecular lattice induced by the motion of magnetic (or electric) fields[195]. Two methods were employed for the T_1 measurements:

a) The inversion recovery method (which is discussed with some detail in section 3.3.2, as it is the one chosen for the present work), and

b) the progressive saturation technique, in which a series of 90° pulses separated by a time

delay τ ($<5T_1$) are applied, i.e. the sequence is $(90^\circ - \tau)_n$, avoiding the long pulse delay of the inversion recovery method.

In method (b) the magnitization will not have time to recover in between pulses, and an equilibrium state will eventually be established[195]. A plot of ln $(M_z - M^0_z)$ against τ will produce a straight line with a gradient $-1/T_1$. Since only a 90° pulse is employed, the peaks are never inverted. In practice, the normal approach when performing progressive saturation measurements is to obtain data at two different τ-values and calculate T_1 from the ratio of the two intensities.

Not a single study was found in the literature for the use of T_1 measurements technique in the investigation of binding of cephalosporins to serum albumin.

T_1 " inversion recovery" and spectrofluorometry "probe displacement" methods are used in the present work.

5.3.1 Theory of Scatchard Plot for calculation of binding parameters

It is assumed that a protein molecule possesses a number of binding sites for a fluorescent probe or drug. If these binding sites have the same affinity for the drug, and are able to act independently (i.e., the binding of a drug molecule to one site does not influence the affinity for the next site), then the law of mass action can be applied to each site individually, and can describe the relationship between binding and the concentration of free drug at equilibrium[157,158] :

$$[P]_f + [D]_f \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [DP] \qquad (5.1)$$

where $[P]_f$ is the molar concentration of unbound protein,

$[D]_f$ is the molar concentration of free drug,

$[DP]$ is the molar concentration of drug-protein complex present,

and k_1 and k_2 are the rate constants for association and dissociation, respectively, of the complex.

At equilibrium, the rates of association and dissociation of the complex are equal, that is :

$$k_1[D]_f[P] = k_2[DP] \tag{5.2}$$

therefore, for each of the binding sites :

$$\frac{k_1}{k_2} = K_a = \frac{[DP]}{[D]_f[P]_f} \tag{5.3}$$

where K_a is the association or affinity constant of the drug for the protein (which is a measure of the affinity of the drug for the protein, with a single binding site).

If there are n independent binding sites, then $n[P]_t$ will be the total concentration of binding sites for the drug, (and since the total concentration of protein $[P]_t$ equals $[P]_f + [DP]$),

$$n[P]_t = [DP] + [P]_f$$

which can be arranged to give

$$[P]_f = n[P]_t - [DP] \tag{5.4}$$

Substitution of equation (5.4) into (5.3) yields

$$K_a = \frac{[DP]}{[D]_f(n[P]_t - [DP])}$$

which may be rearranged,

$$n[D]_f[P]_t - [D]_f[DP] = \frac{[DP]}{K_a}$$

divide by $[P]_t$

$$n[D]_f - \frac{[D]_f[DP]}{[P]_t} = \frac{[DP]}{K_a[P]_t}$$

$$n[D]_f = \frac{[DP]}{K_a[P]_t} + \frac{[D]_f[DP]}{[P]_t}$$

$$= \frac{[DP]}{[P]_t}\left[\frac{1}{K_a} + [D]_f\right]$$

$$= \frac{[DP]}{[P]_t}\left[\frac{1 + [D]_f K_a}{K_a}\right]$$

to give

$$\frac{[DP]}{[P]_t} = \frac{nK_a[D]_f}{1+K_a[D]_f} \qquad (5.5)$$

Now, if r equals moles of drug bound per mole of protein $= \frac{[DP]}{[P]_t}$, then, equation (5.5) may be rewritten as

$$r = \frac{nK_a[D]_f}{1+K_a[D]_f} \qquad (5.6)$$

Hence, on the basis of the law of mass action, it can be deduced that if there are 'n' binding sites per protein molecule, each of which has its own (intrinsic) association constant 'K_{ai}' and if there is no interaction among the bound ions, then the average number of moles of bound drug per mole of protein (r) and its dependence on $[D]_f$ is given by[196]:

$$r = \sum_{i=1}^{i=n} \frac{K_{ai}[D]_f}{1+K_{ai}[D]_f} \qquad (i=1,2,.....n) \qquad (5.7)$$

where K_{ai} is the association or affinity constant for a binding site i.

It has been suggested by Scatchard (1949)[197] for the special case that the K_{ai}'s are equal i.e. all sites are equivalent, equation (5.7) can be rewritten in terms of a single binding constant K_a as

$$r = \frac{nK_a[D]_f}{1+K_a[D]_f} \qquad (5.8)$$

where n is the number of binding sites per protein molecule,
$[D]_f$ is the concentration of free drug,
and K_a is the affinity constant of the drug for the protein.

Rearrangement of equation (5.8) gives the Scatchard equation in its most simple form :

$$\frac{r}{[D]_f} = nK_a - rK_a \qquad (5.9)$$

A plot of the experimental data in terms of $\frac{r}{[D]_f}$ versus r (which is known as a 'Scatchard Plot') will permit the evaluation of n and K_a from the appropriate intercepts (see Fig. 5.2, below).

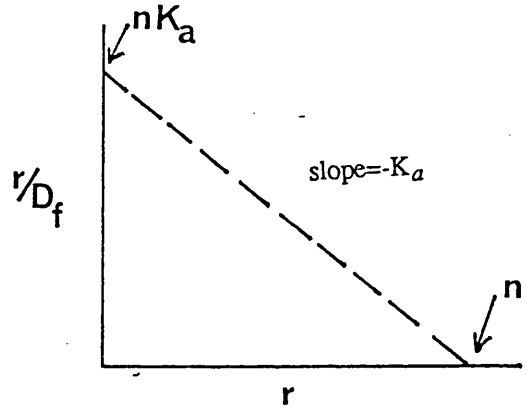


Fig.5.2 : A theoretical Scatchard plot from eq.5.9 (for single class of binding site)[158,160].

From the Scatchard plot, the affinity constant (K_a) of the ligand for the protein can be calculated from the gradient of the graph since

$$[gradient] = -K_a \qquad (5.10)$$

and the number of binding sites (n) obtained from the intercepts of the line with the r-axis (primary sites).

A linear Scatchard plot will only be obtained if a single class of binding sites is involved, each site being totally independent of the others. Deviations from linearity are taken to indicate the involvement of more than one type or class of binding sites or the modification of one protein-binding site by drug interaction at the second site. In practice, Scatchard plots are frequently nonlinear[158].

Klotz (1946)[198], in studying the binding of proteins to small molecules, inverted the law of mass action solved for r to give :

$$\frac{1}{r} = \frac{1+K_a[D]_f}{nK_a[D]_f}$$

$$= \frac{1}{n} + \left(\frac{1}{nK_a[D]_f}\right) \qquad (5.11)$$

A graph of $\frac{1}{r}$ against $\frac{1}{[D]_f}$ is known as 'Klotz Plot' (Fig. 5.3, below), with an intercept of $\frac{1}{n}$ and a slope of $\frac{1}{nK_a}$. This has the disadvantage of concealing deviations from the ideal laws, and of tempting the drawing of straight lines where there should be curvature; it also tends to excessively underemphasise the results obtained at low drug: protein ratios[158]. Hence, it is generally less widely used.

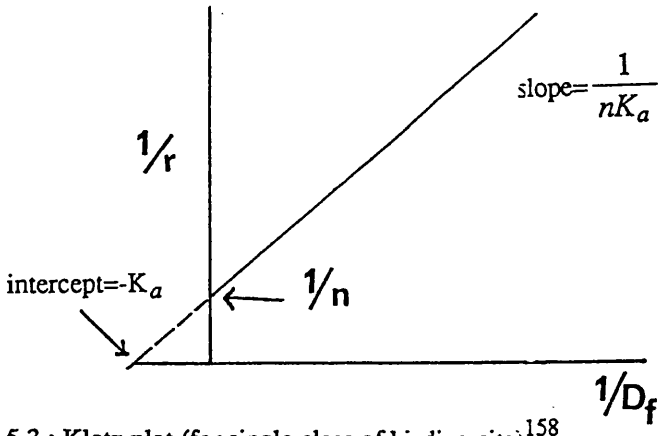


Fig.5.3 : Klotz plot (for single class of binding site)[158].

The Scatchard plot is known to be the most commonly used procedure for the analysis of data on protein-binding, and we have found it convenient to plot our data as suggested by Scatchard[197].

Scatchard theory allows calculation of the affinity constant of the drug in the absence of any interfering compound. An adaptation of equation (5.8) can be applied where there is a competing compound :

$$r = \frac{nK_a[D]_f}{1 + K_a[D]_f + K_{a'}[C]} \qquad (5.12)$$

where $[C]$ is the concentration of the competing compound,
and $K_{a'}$ is the affinity constant of this compound for the protein.

As above, equation (5.12) can be rearranged to give :

$$\frac{r}{[D]_f} = \frac{K_a}{1+K_{a'}[C]}(n-r) \qquad (5.13)$$

A Scatchard plot will therefore allow the calculation of the affinity constant of the competing compound for the protein ($K_{a'}$), assuming that the affinity constant of the drug (K_a) is known, since :

$$[gradient] = \frac{K_a}{1+K_{a'}[C]}$$

and therefore,

$$K_{a'} = -\left(\frac{K_a - [gradient]}{[gradient][C]}\right) \qquad (5.14)$$

This is the method adopted for the calculation of the affinity constants of the cephalosporins for the serum albumins in the present study.

5.3.2 Measurement of drug-protein binding by spectrofluorimetry

5.3.2.1 Spectrofluorimetry theory

Fluorescence spectroscopy is an important tool in the study of the binding of drugs to proteins.

The photoluminescent method of fluorescence is closely related to molecular absorption spectrophotometry. After molecules have absorbed radiant energy and been excited to a higher electronic state, they must lose their excess energy in order to return to the ground electronic state. Fluorescence is the immediate emission (in the order of 10^{-8} sec) of light from a molecule after it has absorbed radiation, lost some of its excitation energy by

vibrations; thus emitted light is always of longer wavelength than that of the exciting light. The phenomenon arises from a singlet-singlet transition (where all of the electrons in the molecule have their spins paired)[191,200]. A schematic energy-level diagram for a diatomic molecule is shown in Fig.5.4 below.

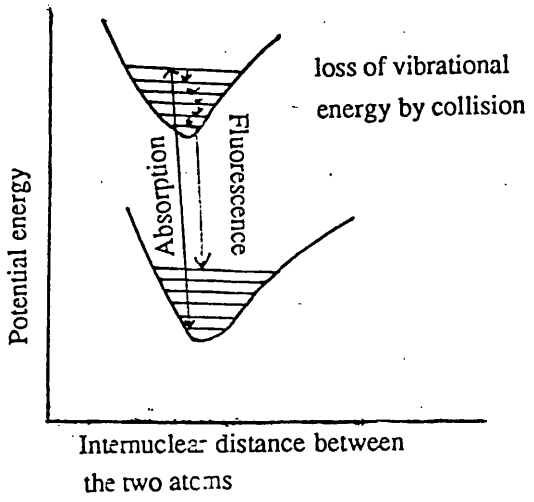


Fig.5.4 : A schematic energy-level diagram for a diatomic molecule[200].

The fluorescence of a given drug molecule may be characterised by several parameters including :

i) the wavelength of maximal excitation and emission (spectra). The former is obtained by setting the emission spectrometer to an emission wavelength of the sample and then scanning a range of wavelengths with the excitation spectrometer. By adjusting the excitation spectrometer to an excitation wavelengths for a sample and causing the emission spectrometer to scan, the emission spectrum of the sample will be recorded.

ii) the quantum yield (Q), which is a measure of the efficiency of fluorescence and is defined as the ratio of the number of photons emitted to the number of photons absorbed (a photon is a quantum of light energy), e.g., Q is zero for nonfluorescent substances.

iii) the fluorescence lifetime, is the time of the fluorescence decay after extinguishing the light source.

iv) the degree of polarization, here the fluorescence of the excited molecules (having their absorbing dipole vectors parallel to the direction of polarized light) will also be polarized to a degree depending on how much Brownian motion has randomized the orientation of these molecules during the excited state lifetime.

These parameters are very sensitive to changes in the molecular environment, e.g. interaction with macromolecules.

5.3.2.2 Choice of technique for fluorescence measurements for studying drug binding

As mentioned in section 5.3.2.1 there are several approaches for studying drug-protein interactions. Fluorescence spectroscopy has been applied under three different conditions :

a) when the drug itself is fluorescent,i.e., any drug that is highly fluorescent both before and after binding to a macromolecule.It has been reported[201] that the binding of the anticoagulant warfarin to HSA results in a sixfold increase in the fluorescent yield of the drug and a small shift in its fluorescence emission maximum to shorter wavelengths.

b) when quenching of protein fluorescence occurs on binding of a drug. Quenching is due to energy transfer from excited-state tryptophan residues to the bound drug. This is illustrated by the effect of thyroxine on serum albumins[202].

c) when the drug itself is not fluorescent but can displace fluorescent probes. This is the method of choice in the present work. It involves the use of fluorescent probes, such as 1-anilinonaphthalene-8-sulfonic acid (ANS), which after binding to a macromolecule, can be used to detect changes in the various structural parameters of the macromolecule.

The use of fluorescent probes has provided insight into the mechanisms and sites of binding of small molecules and has been shown to be a convenient tool for competition studies of small molecules for protein binding sites[203].

A fluorescent probe is defined as a compound that undergoes changes in one or more of its fluorescence properties when bound to certain protein[204]. The observation[203] that certain probes bind to a number of highly hydrophobic protein sites led to the proposition that these compounds might be useful in investigation of the hydrophobic nature of protein[204–206]. An additional observation suggested that the fluorescence intensity of the probes in protein solution is decreased by the introduction of certain drug molecules[181]. This is taken as an indication that competition between drug and probe does occur and that the drug falls into the same type of binding category as characterised by the probe, i.e., binds to hydrophobic binding sites on protein[181]. ANS is the most commonly used fluorescence probe to detect binding at the hydrophobic sites of proteins[175]. Competition with a drug for these sites reduces the fluorescence intensity of the probe-protein complex.

The fluorescence emission spectrum of an aqueous solution of probe in the absence of serum albumin will show a very low fluorescence intensity, but upon binding to the protein molecule the intensity will be strongly enhanced. A change in the nature of fluorescence, i.e., the wavelength of emitted light may also occur. The extent of increase of the probe fluorescence upon addition to serum albumin is used to calculate the binding constants for the albumin-probe complex. The decrease in probe binding caused by a drug and the subsequent decrease in fluorescence of the probe-albumin complex could be used to calculate the binding affinity of the drug for the protein.

5.3.2.3 Theoretical background to fluorescence measurements of drug binding using the probe displacement technique

The fluorescence intensity of a probe, e.g. ANS, which is bound to serum albumin, is a function of the probe concentration, i.e. :

$$[ANS]_b = P \; x \; f \qquad (5.15)$$

where $[ANS]_b$ is the concentration of the probe, ANS, bound to protein,

f is the intensity of the fluorescence produced,

and P is a constant.

The constant, P, is termed the "constant of proportionality" or "the proportionality factor". Hence, if the value of this constant is known, the amount of probe bound to protein, $[ANS]_b$, can be calculated directly from the fluorescence intensity, according to equation (5.15).

To obtain the value of the proportionality constant, P, a solution of probe, ANS, is titrated with a large, 100-fold, exess of protein solution, at which concentration it is assumed that the probe completely bound by protein. It can be seen that as the concentration of protein rises, the fluorescence intensity increases to maximum (f_{max}). At this maximum all the ANS is bound to protein, i.e., $[ANS]_b = [ANS]_t$. Hence, knowing the concentration of the probe and the value of f_{max}, the proportionality constant, P, can be calculated from equation (5.15) to give

$$P = \frac{[ANS]_t}{f_{max}} \qquad (5.16)$$

It should be noted that the observed relative fluorescence intensity, f_o, must be corrected for the " inner filter effect ". This is an attenuation in the intensity of the exciting and emitted beams due to absorption by the sample itself[191]. The correction is made by multiplying the uncorrected fluorescence intensities by the correction factor, x, which is defined as the antilog of the average of the effective optical densities, which are the absorbances at the excitation and emission wavelengths, measured in a 1 cm pathlength cell multiplied by half the width of the fluorescence cell[202]. Hence, for the 1 cm pathlength fluorescence cells used, this factor becomes :

$$x = antilog(\frac{A_{ex} + A_{em}}{2}) \qquad (5.17)$$

where x is the correction factor,

A_{ex} is the absorbance at the excitation wavelength,

and A_{em} is the absorbance at the emission wavelength.

Thus, the corrected fluorescence, f, is defined by

$$f = x \times f_o \qquad (5.18)$$

where f_o is the observed fluorescence intensity obtained directly from fluorimeter reading.

5.3.2.4 Mathematics and application of Scatchard analysis

The fluorescence enhancement of the probe, ANS, upon addition to human (or bovine) serum albumin, can be determined, and the data used to calculate the binding constants for the albumin-probe complex, from a Scatchard plot. The decrease in fluorescence of the albumin-ANS complex in the presence of cephalosporins, can be used to calculate the binding constants of the drug-protein complex.

The fraction of probe bound, $[ANS]_b$, is calculated using the equation (5.15). After the value $[ANS]_b$ is found for each point along the titration curve, the concentration of free probe, $[ANS]_f$, can be calculated if the total concentration of the probe is known since,

$$[ANS]_t = [ANS]_b + [ANS]_f \qquad (5.19)$$

and therefore,

$$[ANS]_f = [ANS]_t - [ANS]_b \qquad (5.20)$$

r can be defined mathematically by,

$$r = \frac{[ANS]_b}{[Protein]_t} \qquad (5.21)$$

(r is the number of moles of ligand bound per mole of protein)

The binding constants (n and K_a) can now be determined from a Scatchard plot of $\frac{r}{[ANS]_f}$ versus r. K_a' for cephalosporins are then measured from equation (5.14, p.205).

5.3.3 Measurement of drug-protein binding by NMR spectroscopy

5.3.3.1 Theoretical basis of NMR relaxation measurements

Relaxation time can be defined as the decay time characterising the return of the nuclei excited by the absorption of radiation to an equilibrium state . It correlates with structural features of molecules, and particularly with their motion.

For purposes of the relaxation experiments to be described in the following sections, a high resolution proton magnetic resonance spectrum can be thought to represent as many spin systems as there are lines in the spectrum. A spin system can be defined as an assembly of nuclei characterised by two energy levels (denoted as α for the lower and β the upper level). When attention is focused on a given spin system, all other nuclei and electrons in the sample are collectively refered to as the lattice. In the presence of electromagnetic radiation of a particular frequency, the spin system absorbs energy from the radiation field, with a consequent increase in the population of the upper state and a corresponding decrease in the population of the lower state. If this absorbed energy is sufficient to equalize the population of the two states, the system is said to have undergone saturation. The return of an either completely or partially saturated system to equilibrium consists of two simultaneously occurring relaxation processes :

1) the spin-spin (or transverse) relaxation, T_2.

2) the spin-lattice (or longitudinal) relaxation, T_1.

Fig. 5.5 (p.212) illustrates the mechanisms of the two relaxation processes. If the state of a spin system is described by its magnetization vector, M, which is the vertical sum of all nuclear magnetic moments comprising the system, i.e., the bulk magnetization vector, then after an applied RF pulse (through an angle Θ degrees) has been removed, the perturbed spin system will begin to relax back towards its equilibrium condition (in which M is aligned along the Z-axis).

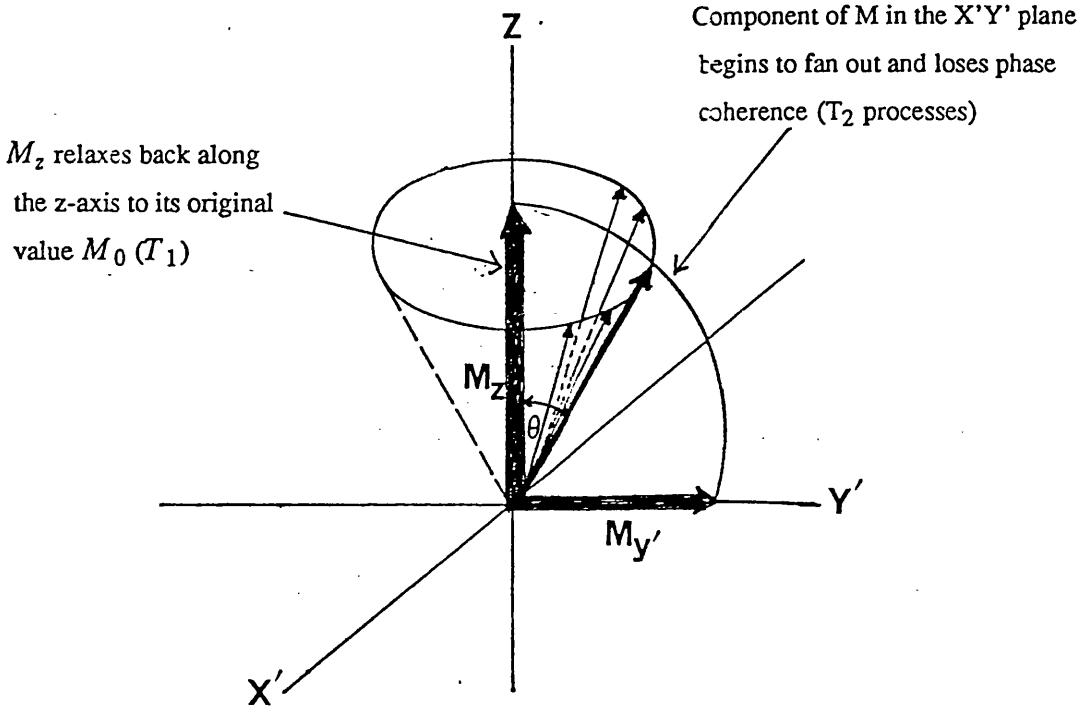


Fig.5.5 : Mechanisms of the two relaxation processes, T_1 and T_2[195].

The spin-spin relaxation process involves the fanning out of the component of M in the X'Y' plane, i.e., it loses coherence with the redistribution of energy among the spin system and there is no detectable signal along the Y' (or the X')-axis. In the spin-lattice relaxation process, the magnetization remaining along the Z-axis relaxes back, along the Z-axis, to its original value M^o, by means of exponential decay characterized by the relaxation time,T_1.

5.3.3.2 Measurement of relaxation time, T_1 bythe inversion recovery method[195,207]

The method involves the application of a 180° pulse to invert the magnetization to the Z-axis. Hence, immediately after the pulse, the magnetization vector M_z equals $-M^0{}_z$. M_z will now begin to relax back along the Z-axis towards its equilibrium value $M^0{}_z$ via the spin-lattice relaxation process (see Fig. 5.6, p.213).

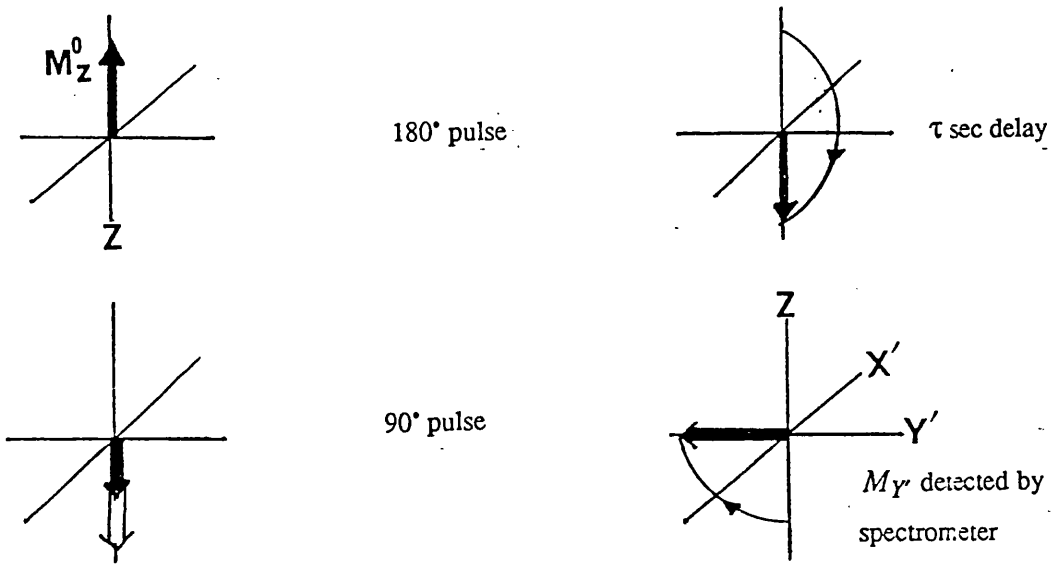


Fig.5.6 : The rotating frame picture of the 180°-τ-90° pulse experiment.

As most spectrometers do not detect signals along the Z-axis, after the 180° pulse, a second 90° pulse is applied after a delay of τ sec which tips the magnetization onto the -Y'-axis, where it can be detected, i.e., the full sequence is : 180° - delay τ - 90° pulse. At the end of the cycle the magnetization lies along the -Y'-axis and so, before a second 180° pulse can be applied, it is necessary to allow a period $5T_1$ sec (where T_1 is the longest in molecule) to allow the magnetization vector to relax back to M^0_z. (It is also assumed that during a $5T_1$ delay, the transverse magnetization "T_2" is allowed to decay completely[9]). Hence, a pulse delay (PD) equal to $5T_1$ must be inserted between each cycle, giving the overall sequence $(180° - \tau - 90° - PD)_n$, where n is the number of times the cycle is repeated. The timing of the pulses and delays is normally formed automatically by the computer. At the end of the experiment the accumulated FID (Free Induction Decay) is transformed and the intensity of the signal (which is a function of T_1) determined. This can be expressed mathematically as :

$$M_z = M^0{}_z(1 - 2exp\frac{-t}{T_1}) \qquad (5.22)$$

where M_z is the component of M along the Z-axis t sec after the application of the 180° pulse, $M^0{}_z$ is its equilibrium value, and T_1 is the spin-lattice relaxation time.

Rearranging equation (5.22) gives

$$M_z - M^0{}_z = -2M^0{}_z \exp\frac{-t}{T_1} \qquad (5.23)$$

Hence, taking logs,

$$ln(M_z - M^0{}_z) = ln(2M^0{}_z) - \frac{t}{T_1} \qquad (5.24)$$

Hence, a plot of $\ln(M_z - M^0{}_z)$ against τ will give a staight line with a gradient of - $\frac{1}{T_1}$.

In practice, a series of cycles with different values of τ are carried out and the value of $M^0{}_z$ determined by using one cycle in which $\tau > 5T_1$. A graph of $\ln(M_z - M^0{}_z)$ is then plotted as a function of τ and the value of T_1 determined.

5.4 Experimental

5.4.1 Spectrofluorimetry

5.4.1.1 Instrumentation

1) Fluorimetric measurements were made at 24°C with a Shimadzu microcomputer-controlled recording spectrofluorophotometer model RF-540 (Kyoto, Japan), equipped with a 150 W xenon lamp, a monitoring photomultiplier R212-09, and a photometric photomultiplier R452-01 tube. Measuring conditions can be stored and printed with a Shimadzu data recorder unit model DR-3. The entrance slit for the excitation light and the exit slit for emission light were both 10 nm. The relative fluorescence intensities of bound ligand were obtained directly from fluorometer readings of uncorrected excitation and emission wavelengths.

2) Optical densities were measured in a Perkin Elmer Lambda 3 UV/VIS spectro photometer. Absorption readings were taken at the excitation and emission wavelengths (both previously determined from fluorescence spectra of a solution of serum albumin and probe).

3) pH measurements were made in a Kent EIL 7020 pH meter.

4) Stirring, during fluorometric titrations, was achieved with a small magnetic flea by means of a magnetic stirrer.

5) SGE microlitre syringes, Scientific Glass Engineering PTY. Ltd., Ringwood, Australia.

5.4.1.2 Materials

The following materials were used without further purification :

1. Cephalosporin derivatives studied were: cephalothin Na, cephazolin Na, cefatrizine, cephalexin, cefotaxime Na, desacetylcefotaxime, and cefotaxime lactone. (list of suppliers in Chapter Two, Table 2.1).

2. Bovine serum albumin (BSA) : Albumin, Bovine powder fraction V, 96-99% albumin, Sigma Chemicals Co., USA. A molecular weight of 68,000 was assumed for this lot.

3. Human serum albumin (HSA) : Albumin, Human No. A-1887, fraction V, molecular weight 69,000. Sigma Chemicals Co., USA.

4. 8-Anilino-1-naphthalenesulfonic acid, ammonium salt (F.W.=316.38), melting point 242-244°C. Aldrich Chemicals Co., England.

5. Highly purified water (Milli-Q water systems, Millipore S.A, France).

6. All other chemicals were of reagent grade and used without further purification.

5.4.1.3 Preparation of phosphate buffer of pH 7.4 and ionic strength (I) of 0.154 at room temperature

The buffer was prepared by mixing appropriate amounts of 0.1 M disodium hydrogen orthophosphate (Na_2HPO_4) and 0.1 M potassium dihydrogen orthophosphate (KH_2PO_4) to pH 7.4. The ionic strength was adjusted to 0.154 by 0.9% sodium chloride according to the

following equation[209]:

$$I = 0.5 \sum C_i X Z^2_i \qquad (5.25)$$

where C_i is the molar concentration of the ion species i,
and Z_i is the valency of ion species i.

5.4.1.4 Experimental conditions

(i) All solutions were freshly prepared in phosphate buffer.

(ii) The serum albumin powder was dried overnight under vacuum before use.

(iii) The serum albumin solutions were stored, foil wrapped in refrigerator overnight, before they were used for binding studies, so all the molecules had become fully hydrated and had taken up a stable conformation, and to allow the fluorescence to stabilize.

(iv) Titrations with ANS were carried out at both low and high serum albumin concentrations.

(v) All titrations performed at ~ 24°C.

(vi) The following concentrations were used for the titrations :

a) for ANS : 1 x 10^{-3}M.

b) for serum albumin : 1 x 10^{-5}M and 1 x 10^{-6}M.

c) for cephalosporins : 5 x 10^{-4}M.

(vii) The binding data were the mean of at least two measurements.

(viii) In order to avoid variations in the buffer pH, probe and albumin concentrations, all the cephalosporin solutions for a particular group of titrations were made at the same time. These solutions were used within 24 hours to avoid errors due to any degradations of the cephalosporins.

5.4.1.5 Methods

The binding of the probe, ANS, to serum albumin was determined by measuring the increase in fluorescence following titration of the protein solution with the probe as described

by Brand et al.[203] and Hsu, Ma, and Jun[208] and modified as described below.

Fluorescence measurements were made at 478 - 480 nm using an excitation wavelength of 419 nm at high (1 X 10^{-5}M) serum albumin concentration, and at 487 nm using an excitation wavelength of 419 nm at low (1 X 10^{-6} M) serum albumin concentration. All measurements were carried out at (~ 24°C). Solutions of serum albumin were prepared in buffer. The probe, ANS, was in buffer at a concentration of 1 X 10^{-3}M.

The above-mentioned instrumental conditions set-up (performed by fluorimetric scans for probe-protein solutions) were kept constant throughout all titrations carried out as in Table 5.4 (p.218).

1) For determination of proportionality constant (*P*)

The constant *P* was determined from titrations A and B (Table 5.4, p.218) as follows:

3 ml of the probe solution was pipetted into a fluorometer sample cell of one cm pathlength. A small magnetic flea was then introduced into the cell. Initial measurements of fluorescence and optical densities were then taken, separately. Successive addition of protein solution, in increments of 3, 5, 10 and 20 µl, was continued until obtaining an almost constant fluoresence intensity reading. The contents of cell were allowed to stir for at least 40 sec after each addition of protein solution increments and before any measurements were taken. Additions were made by means of SGE microsyringes.

To minimise any photodecomposition of solution during fluorescence titrations, the contents of the sample cell were exposed to light of the excitation wavelength only during the actual measurement of fluorescence intensity. Furthermore, the instrumental setting conditions were kept constant throughout the titration.

The above titration of probe with serum albumin was used to determine the limiting fluorescence when this concentration of probe was completely bound. This value was then used to calculate the proportionality factor (eq.5.4, p.201), and accordingly, the concentrations of free and bound probe in a reverse titration of serum albumin with probe.

Table 5.4 : Titrations of ANS/ HSA (or BSA) in absence and presence of cephalosporin derivatives

A) 3 ml of 1.0 X 10^{-5}M ANS was titrated with a solution of 1.0 X 10^{-3}M BSA (in 1.0 X 10^{-5}M ANS).

B) 3 ml of 5.0 X 10^{-6}M ANS was titrated with a solution of 5.0 X 10^{-4}M HSA (in 5.0 X 10^{-6}M ANS).

C) 3 ml of 1.0 X 10^{-5}M BSA was titrated with a solution of 1.0 X 10^{-3}M ANS (in 1.0 X 10^{-5}M BSA).

D) 3 ml of 2.76 X 10^{-6}M BSA was titrated with a solution of 1.0 X 10^{-3}M ANS (in 2.76 X 10^{-6}M BSA).

E) 3 ml of 1.0 X 10^{-6}M HSA was titrated with a solution of 1.0 X 10^{-3} ANS (in 1.0 X 10^{-6}M HSA).

F) 3 ml of 5.0 X 10^{-4}M cephalosporin (in 2.76 X 10^{-6}M BSA) was titrated with a solution of 1.0 X 10^{-3}M ANS (in 5.0 X 10^{-4}M cephalosporin in 2.76 X 10^{-6}M BSA).

G) 3 ml of 5.0 X 10^{-4}M cephalosporin (in 1.0 X 10^{-6}M HSA) was titrated with a solution of 1.0 X 10^{-3}M ANS (in 5.0 X 10^{-4}M cephalosporin in 1.0 X 10^{-6}M HSA).

The solutions of the probe were made up in a solution containing the same concentration of HSA or BSA (and cephalosporin) as in the solution being titrated so that these concentrations would not vary during the titration. Similarly for titrations A and B, the protein solutions were made up in ANS solution.

2) For determination of binding parameters of ANS to serum albumin (from titrations C to E)

The fluorescence of ANS is enhanced on binding to serum albumin, and the emission maximum is blue-shifted (from 512 nm to 480 nm). The fluorescence intensities of the protein-probe complex as a function of probe concentration (1 X 10^{-3}M) were measured at the protein concentrations, (1 X 10^{-6}M) and (1 X 10^{-5}M) in phosphate buffer. The titration steps were similar to those used for determination of P, except that the titrant this time was the probe solution : 3 ml of each protein solution was titrated with successive additions of 3, 5, 10 and 20 µl of probe solution. Enhancement of the probe's fluorescence upon addition to serum albumin, was determined, and these data were used to calculate the binding constant for the albumin-probe complex.

3) Displacement of ANS from serum albumin by cephalosporin derivatives (titrations F and G)

Cephalosporin binding was determined by repeating the titrations of 3 ml low protein solutions with probe in the presence of 5.0 X 10^{-4}M cephalosporin. The decrease in fluorescence of ANS-albumin complex in presence of cephalosporins could be used to calculate the binding constants of the drug.

(N.B. The instrument was zeroed before use with reference solvent).

5.4.1.6 Calculation of [ANS]$_f$

The total ANS concentration is calculated as follows : In the titration of solutions of 1.0 X 10^{-5}M serum albumin with 1.0 X 10^{-3}M ANS, assuming x µl is the increment of ANS added to the 3 ml of protein solution in the fluorescent cell, then, after this addition, the total

The solutions of the probe were made up in a solution containing the same concentration of HSA or BSA (and cephalosporin) as in the solution being titrated so that these concentrations would not vary during the titration. Similarly for titrations A and B, the protein solutions were made up in ANS solution.

2) For determination of binding parameters of ANS to serum albumin (from titrations C to E)

The fluorescence of ANS is enhanced on binding to serum albumin, and the emission maximum is blue-shifted (from 512 nm to 480 nm). The fluorescence intensities of the protein-probe complex as a function of probe concentration (1 X 10^{-3}M) were measured at the protein concentrations, (1 X 10^{-6}M) and (1 X 10^{-5}M) in phosphate buffer. The titration steps were similar to those used for determination of *P*, except that the titrant this time was the probe solution : 3 ml of each protein solution was titrated with successive additions of 3, 5, 10 and 20 µl of probe solution. Enhancement of the probe's fluorescence upon addition to serum albumin, was determined, and these data were used to calculate the binding constant for the albumin-probe complex.

3) Displacement of ANS from serum albumin by cephalosporin derivatives (titrations F and G)

Cephalosporin binding was determined by repeating the titrations of 3 ml low protein solutions with probe in the presence of 5.0 X 10^{-4}M cephalosporin. The decrease in fluorescence of ANS-albumin complex in presence of cephalosporins could be used to calculate the binding constants of the drug.

(N.B. The instrument was zeroed before use with reference solvent).

5.4.1.6 Calculation of [ANS]$_t$

The total ANS concentration is calculated as follows : In the titration of solutions of 1.0 X 10^{-5}M serum albumin with 1.0 X 10^{-3}M ANS, assuming x µl is the increment of ANS added to the 3 ml of protein solution in the fluorescent cell, then, after this addition, the total

volume in the cell = (3000 µl + x µl); and as x µl of 1.0 X 10^{-3}M ANS=(x X 100) µl of 1.0 X 10^{-5}M. Therefore, the total ANS concentration in the cell will be

$$[ANS]_t = \frac{x\,X\,100}{3000 + x} 10^{-5} M \qquad (5.26)$$

With low protein concentration (10^{-6}M)

$$[ANS]_t = \frac{x\,X\,1000}{3000 + x} 10^{-6} M \qquad (5.27)$$

5.4.2 NMR

5.4.2.1 Instrumentation

The determination of relaxation times (T_1) were performed on a Jeol GX270 FT NMR spectrometer operating at 270 MHz with 28 µs (180°) and 14 µs (90°) pulses, 3001 Hz spectral width and acquisition time of 2.73 s. Magnetization decay obtained in the detection mode was accumulated on 16 K memory and sensitivity enhancement of 0.1 Hz was applied before the Fourier transformation.

5.4.2.2 Materials

The materials used were the same as in section 5.4.1.2, in addition to the following :

1) Cephaloridine and cephalexin (Glaxo, U.K.), and cefadroxil (Latina, Italy).
2) Benzylpenicillin Na (Glaxo, U.K.).
3) Deuterium oxide (D_2O), 99.9 Atom%D, MSD, Montreal, Canada.

5.4.2.3 Experimental conditions

1. Unless otherwise specified, all experiments were conducted in deuterated 0.05M phosphate buffer, pH 7.4 and I=0.154, at room temperature (see section 5.4.1.3 for preparation of buffer, using D_2O instead of distilled water). The pH values of the buffer are actual meter reading and are corrected for deuterium isotope effects, i.e., pH meter (7.4) + 0.4[209].

2. Drug concentrations are all expressed as molar (M), and protein concentrations are given in percent weight per volume (% w/v).

3. All drug solutions were freshly prepared in the deuterated buffer before use. Drug-protein solutions were made up by adding the freshly prepared drug solution to weighed amounts of serum albumin immediately before the spectra were recorded.

4. The final concentration of protein was in the range 0 - 10 % w/v (0 - ~ 1.5 X 10^{-3}M), based upon a molecular weight of 69,000.

5. All measurements were made within 5 hours. No change was observed for periods as long as 8 hours.

6. Deuterium in the solvent was used as a lock signal.

7. Solvent elimination : The presence of a large peak at 4.8 ppm due to the residual HOD can cause considerable problems because it obscures part of the spectrum and restricts dynamic range. The solvent peak was eliminated by gated secondary irradiation such that the decoupler was on only during the pulse delay (homo- gated decoupling)[195].

5.4.2.4 Methods

The longitudinal relaxation times (T_1) of solutions of cephalosporins in 0.05 M deuterated phosphate buffer were determined either in absence or presence of added serum albumin.

As already mentioned in section 5.3.3.2 the mesurements of T_1 were performed by using the $(180° - \tau - 90° - PD)_n$ sequence. In our experiments, a series of cycles with different values of τ, ranging from 0.005 to 25 sec and an average PD value of 25 sec, were carried out. At the end of the experiment the accumulated FID was transformed; the T_1 values of the protons were determined by least-squares regression of the relation $\ln(M_z - M^0{}_z) = \ln(2M^0{}_z) - \frac{\tau}{T_1}$, and the results were output directly on the teletype. The spectra were output as a 'stacked plot'.

5.5 Results

5.5.1 Spectrofluorimetry

The results documented here were the mean values of at least two measurements. The standard deviation for the fluorimetric measurements was less than 7 % of the mean.

The excitation and emission spectra of a) 1.0 X 10^{-3} M ANS. b) 1.0 X 10^{-3} M ANS in 1.0 X 10^{-5} M HSA, measured in pH 7.4 phosphate buffer are shown in Fig. 5.7 (p.225). The enhancement of the fluorescence intensity of ANS on binding to serum albumin appears clearly, accompanied by a blue shift of the emission wavelength maximum from 512 nm to ~ 488 nm.

5.5.1.1 Interactions with HSA

A) Determination of the proportionality factor, P

The value of (P) was determined from titration B (Table 5.4, p.218), and was used in the treatment of the results from other titrations. The concentration of ANS used was 5.0 X 10^{-6}M, and f_{max} was 73.5 (as obtained from Fig. 5.8, p.226); hence, according to equation (5.16, p.209),

$$P = \frac{5.0\,X\,10^{-6}}{73.5} = 6.80\,X\,10^{-8} M \pm 0.21$$

This P value was only correct for the sensitivity settings used during its determination titration; it represented the mean value of four separate measurements.

B) Binding of ANS to HSA

Table 5.5 (p.227) lists the mean complete data as obtained from the fluorimetric and UV/VIS absorption titrations of 1.0 X 10^{-6} M HSA with a solution of 1.0 X 10^{-3} M ANS (titration E in Table 5.4, p.218), in addition to the calculated values of the free, bound and total ANS concentrations, r and $\frac{r}{[ANS]_f}$.

The binding constants of ANS for HSA were calculated from the Scatchard plot (Fig. 5.9a, p.230) of $\frac{r}{[ANS]_f}$ versus r, as reported in section 5.4.1.5, where K_a is the affinity constant of ANS for HSA, and the number of moles of ANS bound per mole of HSA (r) is an expression of the number of binding sites, n, on a molecule of HSA,

hence,

$$K_a = slope\ of\ curve$$

$$= \frac{0.94}{0.28} X 10^6 M^{-1}$$

$$= 3.36 X 10^6 M^{-1}$$

and n (intercept of the line on r-axis) = 1.37

C) Displacement of ANS from HSA by cephalosporins

Table 5.6 (p.228) presents data for corrected fluorescence intensities of ANS in absence and in presence of examined cephalosporins : cephalothin Na, cephazolin Na, cephalexin, cefotaxime Na, desacetylcefotaxime, and cefotaxime lactone.

Competition between ANS and the cephalosporins for the binding sites in proteins (as evidenced by the decrease of fluorescence intensities of ANS in presence of the examined cephalosporins, Table 5.6), can be quantitated by the calculations reported below.

An example calculation of the binding constants of cephalothin for HSA is presented below :

The binding parameters ($K_{a'}$ and n) for cephalothin Na were determined from the data in Table 5.7 (p.229) and the Scatchard plot illustrated in Fig.5.9**e** (p.230), and according to equation (5.14), the affinity constant ($K_{a'}$) of cephalothin for HSA was calculated from the gradient of the graph (Fig. 5.9**e**) as follows :

$$gradient\ of\ line = \frac{2.01-1.70}{0.5-0.4} X 10^5 M^{-1}$$

$$= 3.1X10^5 M^{-1}$$

$$K_a = 3.357X10^6 M^{-1} \textbf{ (as determined in previous section)}$$

$$\textbf{and } [C] = 5.0X10^{-4} \textbf{ M (from Table 5.4)}$$

Hence,

$$K_{a'} = \frac{(33.57-3.10)X10^5}{3.1X10^5 X 5.0X10^{-4}}$$

$$= 1.966 \times 10^4$$

The number of binding sites (n) was obtained from the intercept of the line with the r-axis,

n = 1.1

Fig. 5.9 (p.230) reports the Scatchard plots obtained for ANS alone and in the presence of examined cephalosporins. The intercepts on the abscissa are almost identical (n = 1.0 - 1.4) for the five examined compounds thus indicating a competition between ANS and the drugs at the same, or adjacent, sites. Furthermore, the slopes of curves are different for the different compounds under study and the calculated relative binding constants are summarised in Table 5.8 (p.231), with comparison to those obtained by other workers.

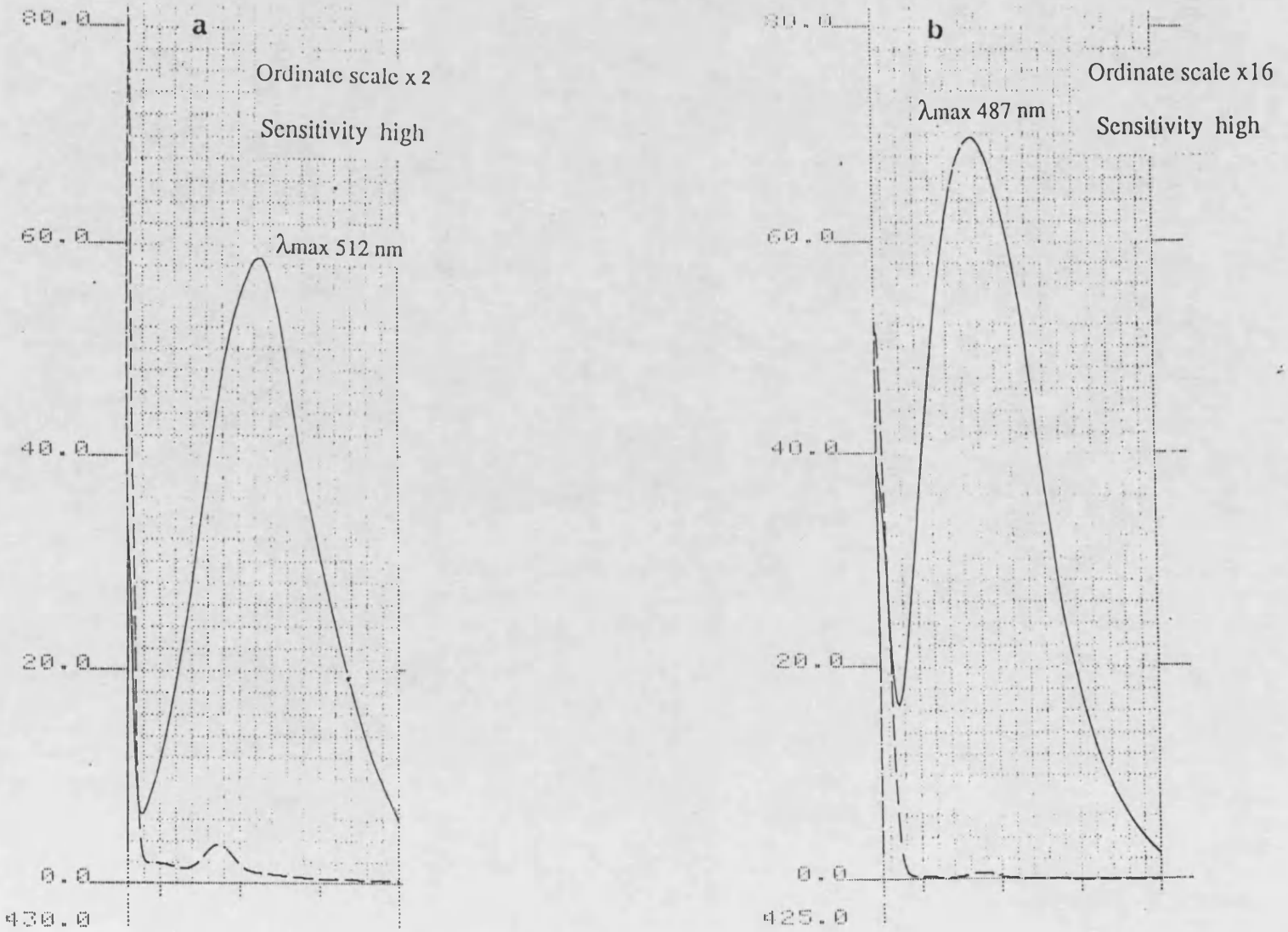


Fig.5.7 : Fluorescence emission spectra of ANS (at excitation wavelength of 418 nm), 1 x 10^{-3} M. in phosphate buffer (0.05 M) pH 7.4 and I 0.154 in absence (a) and in presence (b) of 1 x 10^{-6} M HSA.

Instrumental settings are indicated in the figures

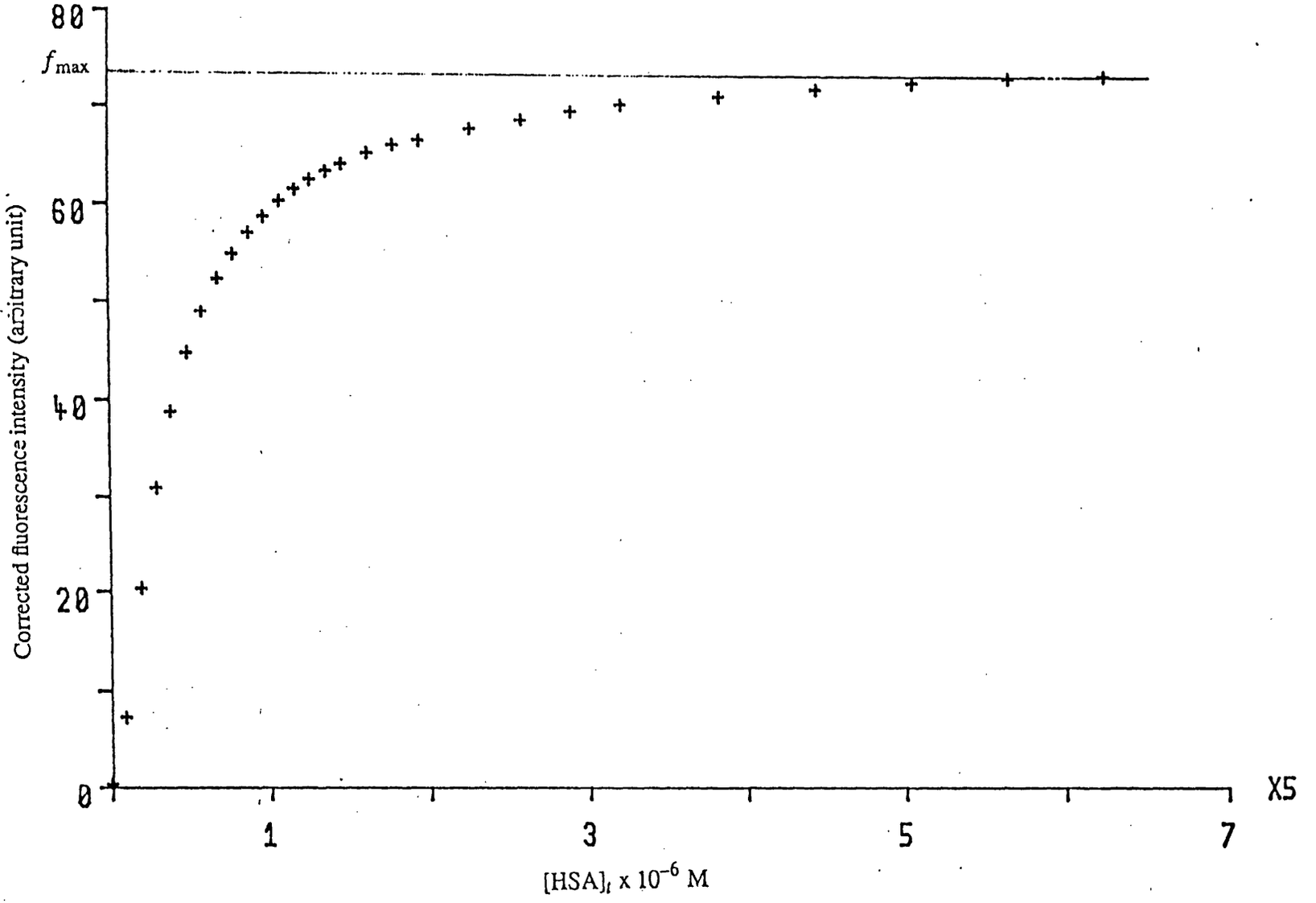


Fig.5.8 : Plot of corrected fluorescence intensity vs $[HSA]_t$, for the calculation of the proportionality factor, P.

Table 5.5[a] : Data for the calculation of binding parameters of ANS for HSA from a Scatchard plot of $\frac{r}{[ANS]_f}$ vs r

µl of ANS added[c]	fo	UV abs[b] λex	X	f	$[ANS]_t$ x 10^{-6}	$[ANS]_b$ x 10^{-6}	$[ANS]_f$ x 10^{-6}	r	$\frac{r}{[ANS]_f}$
3	8.78	.009	1.017	8.93	1.0	.61	0.39	.61	1.55
6	13.55	.010	1.018	13.8	2.0	.94	1.06	.94	0.88
9	16.75	.011	1.019	17.07	3.0	1.16	1.84	1.16	0.63
12	19.08	0.0115	1.02	19.46	3.98	1.32	2.65	1.32	0.50
15	21.03	0.012	1.021	21.47	4.98	1.46	3.52	1.46	0.42
18	22.63	0.0125	1.022	23.12	5.96	1.57	4.39	1.57	0.36
21	23.98	0.0133	1.022	24.52	6.95	1.67	5.28	1.67	0.32
24	25.20	0.014	1.023	25.78	7.94	1.75	6.19	1.75	0.28
27	26.25	0.0143	1.024	26.87	8.92	1.83	7.09	1.83	0.26
30	27.23	0.0145	1.024	27.88	9.90	1.90	8.00	1.90	0.24
35	28.63	0.015	1.025	29.33	11.53	1.99	9.53	1.99	0.21
40	29.90	0.0155	1.025	30.65	13.16	2.08	11.08	2.08	0.19
45	31.00	0.016	1.026	31.80	14.78	2.16	12.62	2.16	0.17
50	31.93	0.018	1.027	32.81	16.40	2.23	14.17	2.23	0.16
60	33.85	0.019	1.030	34.85	19.61	2.37	17.24	2.37	0.14
80	36.78	0.022	1.033	37.97	25 97	2.58	23.39	2.58	0.11

fo=observed fluorescence intensity, f=corrected fluorescence intensity = fo x P, X=correction factor,

b λex=419 nm, absorbance at λem487 nm = 0.006 (constant)

c [ANS]=1 x 10^{-3}M,

$[HSA]_t$=1 x 10^{-6}M, P=0.068 x 10^{-6}M

Table 5.5[a] : Data for the calculation of binding parameters of ANS for HSA from a Scatchard plot of $\frac{r}{[ANS]_f}$ vs r

µl of ANS added[c]	fo	UV abs[b] λex	X	f	$[ANS]_t$ $\times 10^{-6}$	$[ANS]_b$ $\times 10^{-6}$	$[ANS]_f$ $\times 10^{-6}$	r	$\frac{r}{[ANS]_f}$
3	8.78	.009	1.017	8.93	1.0	.61	0.39	.61	1.55
6	13.55	.010	1.018	13.8	2.0	.94	1.06	.94	0.88
9	16.75	.011	1.019	17.07	3.0	1.16	1.84	1.16	0.63
12	19.08	0.0115	1.02	19.46	3.98	1.32	2.65	1.32	0.50
15	21.03	0.012	1.021	21.47	4.98	1.46	3.52	1.46	0.42
18	22.63	0.0125	1.022	23.12	5.96	1.57	4.39	1.57	0.36
21	23.98	0.0133	1.022	24.52	6.95	1.67	5.28	1.67	0.32
24	25.20	0.014	1.023	25.78	7.94	1.75	6.19	1.75	0.28
27	26.25	0.0143	1.024	26.87	8.92	1.83	7.09	1.83	0.26
30	27.23	0.0145	1.024	27.88	9.90	1.90	8.00	1.90	0.24
35	28.63	0.015	1.025	29.33	11.53	1.99	9.53	1.99	0.21
40	29.90	0.0155	1.025	30.65	13.16	2.08	11.08	2.08	0.19
45	31.00	0.016	1.026	31.80	14.78	2.16	12.62	2.16	0.17
50	31.93	0.018	1.027	32.81	16.40	2.23	14.17	2.23	0.16
60	33.85	0.019	1.030	34.85	19.61	2.37	17.24	2.37	0.14
80	36.78	0.022	1.033	37.97	25 97	2.58	23.39	2.58	0.11

fo=observed fluorescence intensity, f=corrected fluorescence intensity = fo x P, X=correction factor,

b λex=419 nm, absorbance at λem487 nm = 0.006 (constant)

c. [ANS]=1 x 10^{-3}M,

$[HSA]_t$=1 x 10^{-6}M, P=0.068 x 10^{-6}M

HSA

Table 5.6 : Corrected fluorescence intensities of ANS in absence and in presence of examined cephalosporins[a]

µl ANS *added*[b]	$[ANS]_t$ 10^{-6}	Corrected fluorescence intensity (arbitrary unit)						
		ANS	CETH	CEZ	CEX	CEF	DesCEF	CEFlact
3	1.0	8.93	3.41	5.83	7.01	7.16	6.59	4.96
6	2.0	13.80	5.27	8.91	10.68	10.95	9.70	8.71
9	3.0	17.07	6.72	11.17	13.23	13.82	11.78	11.65
12	3.98	19.46	8.07	13.04	15.19	15.97	13.35	13.87
15	4.98	21.47	9.43	14.59	16.84	17.83	14.80	15.90
18	5.96	23.12	10.16	16.05	18.27	19.27	16.04	17.54
21	6.95	24.52	11.20	17.19	19.41	20.52	17.19	19.06
24	7.94	25.78	12.13	18.24	20.57	21.56	18.03	20.30
27	8.92	26.87	13.45	19.18	21.71	22.70	18.97	21.52
30	9.90	27.88	13.70	20.14	22.54	23.53	19.81	22.56
35	11.53	29.33	14.74	21.49	24.00	24.89	20.98	24.09
40	13.16	30.65	15.89	22.63	25.17	26.14	21.94	25.44
45	14.78	31.80	16.85	23.68	26.22	27.20	23.00	26.69
50	16.40	32.81	17.81	24.74	27.27	28.15	23.87	27.74
60	19.61	34.85	19.84	27.00	29.26	30.07	25.60	29.85
70	22.80	36.54	21.55	28.78	30.86	31.55	27.11	31.62
80	25.97	37.97	23.18	30.37	32.26	32.94	28.31	32.99
100	32.26	40.25	25.85	33.04	34.63	35.14	30.41	35.30
120	38.46	42.10	28.10	35.37	36.50	36.94	32.32	37.21
140	44.59	43.71	30.06	37.36	38.19	38.40	33.80	38.75

a CETH=cephalothin, CEZ=cephazolin, CEX=cephalexin, CEF=cefotaxime, DesCEF=desacetylcefotaxime, CEFlact=cefotaxime lactone, b ANS concentration=1 x 10^{-3}M

Table 5.7 : Data for the calculation of the binding parameters of cephalothin for HSA from a Scatchard plot of $\frac{r}{[ANS]_f}$ vs r

Corrected fluorescence intensity, F	$[ANS]_t$ x 10^{-6}	$[ANS]_b$ x 10^{-6} P x F	$[ANS]_f$ x 10^{-6}	$r = \frac{[ANS]_b}{[HSA]_t}$	$\frac{r}{[ANS]_f} \times 10^{-6}$
1.2	0.2	0.0816	0.1184	0.0816	0.689
1.76	0.4	0.1197	0.2803	0.1197	0.427
2.26	0.6	0.1537	0.4463	0.1537	0.344
2.72	0.8	0.1850	0.6150	0.1850	0.301
3.20	1.0	0.2176	0.7824	0.2176	0.278
3.60	1.2	0.2448	0.9552	0.2448	0.256
4.00	1.4	0.2720	1.1280	0.2720	0.241
4.40	1.6	0.2992	1.3008	0.2992	0.230
4.70	1.8	0.3196	1.4804	0.3196	0.216
5.10	2.0	0.3468	1.6532	0.3468	0.210
5.76	2.4	0.3917	2.0083	0.3917	0.195
6.40	2.8	0.4352	2.3648	0.4352	0.184
7.00	3.2	0.4760	2.7240	0.4760	0.175
7.52	3.6	0.5114	3.0886	0.5114	0.1656
8.04	4.0	0.5467	3.4533	0.5467	0.158
8.80	4.6	0.5984	4.0016	0.5984	0.150
9.50	5.2	0.6460	4.5540	0.6460	0.142
10.16	5.8	0.6909	5.1091	0.6909	0.1352
10.78	6.4	0.7330	5.6670	0.7330	0.129
11.36	7.0	0.7725	6.2275	0.7725	0.124
12.20	8.0	0.8296	7.1704	0.8296	0.116
13.00	9.0	0.8840	8.1160	0.8840	0.109
13.80	10.0	0.9384	9.0616	0.9384	0.104
15.20	12.0	1.0336	10.9664	1.0336	0.094
16.44	14.0	1.1179	12.8821	1.1179	0.087
17.70	16.0	1.2036	14.7964	1.2036	0.081
20.00	20.0	1.3600	18.6400	1.3600	0.073

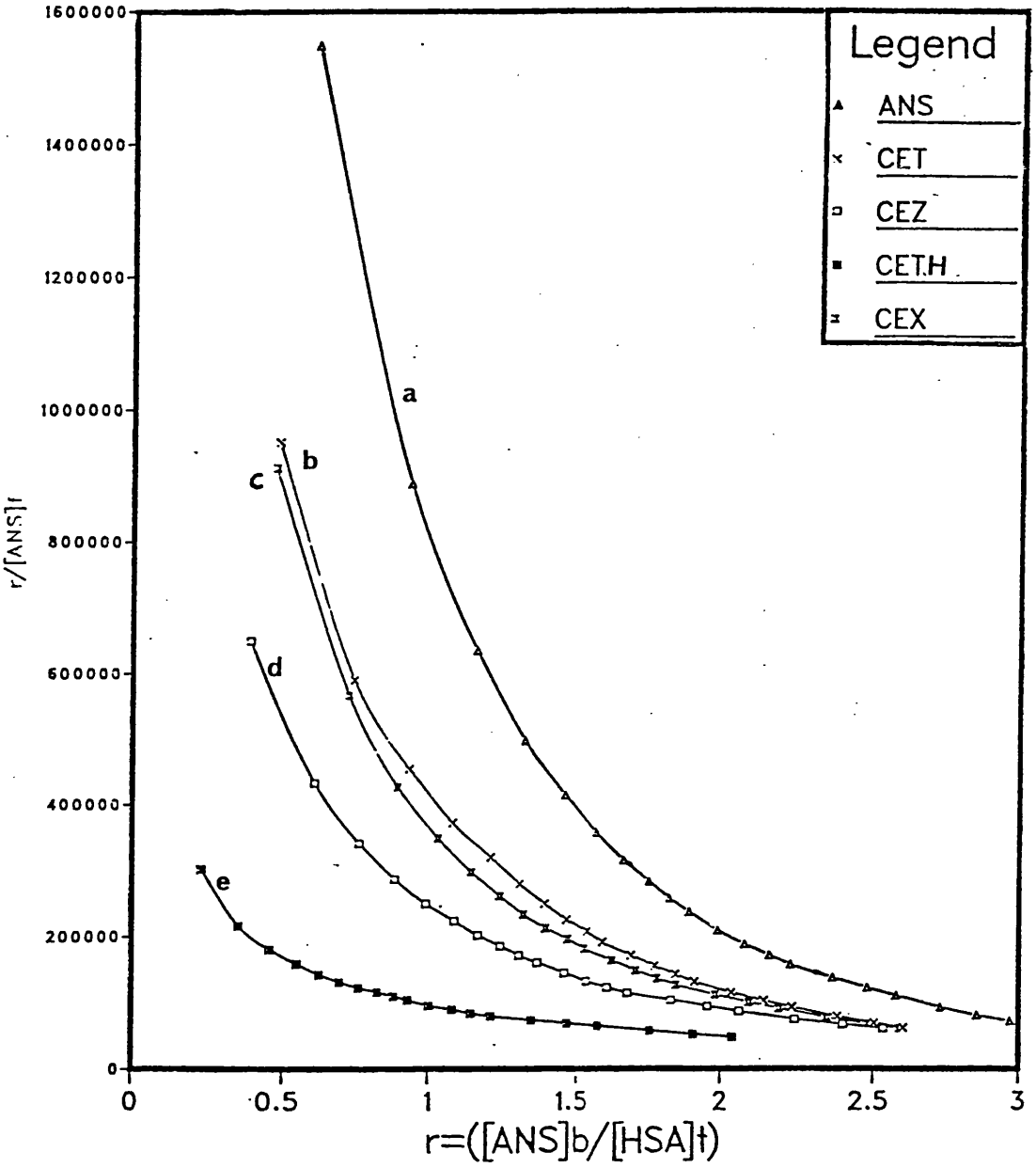


Fig.5.9 : Scatchard plot of ANS for HSA in absence (a) and in presence of the examined cephalosporins (b-e).

ANS= 8-anilino -1-naphthalenesulphonic acid, ammonium salt, CET=cefotaxime Na, CEZ=cephazolin, CETH=cephalothin, CEX=cefalexin.

Table 5.8 : Summary of binding parameters of studied cephalosporins to HSA (with comparison to those of other workers)

Cephalosporin	Present work K_a or $K_{a'}$ (M^{-1}) n	Csiba[179]	Kim[186]	Veronese[180]
ANS	3.36×10^6 1.37	1.38×10^6 1.4		1.2×10^6 2.1
Cephalothin	19.7×10^3 1.1	4×10^5 1.4	3.3×10^3 3.0	0.9×10^3 2.1
Cephazolin	5.37×10^3 1.1	3.7×10^5 1.4	6.36×10^3 3.0	
Cephalexin	1.72×10^3 1.1	8.6×10^5 1.4		0.4×10^3 2.1
Cefotaxime	2.83×10^3 1.2	8.1×10^5 1.4	6.6×10^3 3.0	
Desacetyl cefotaxime	1.89×10^3 1.1			
Cefotaxime lactone	10.05×10^3 1.7			

5.5.1.2 Interaction with BSA

A) Determination of proportionality factor, *P*

The value of the proportionality factor (*P*) was determined from Fig. 5.10 (p.233) as follows :

$$P = \frac{[ANS]_t}{f_{max}}$$

$$= \frac{1.0X10^{-5}}{94.7} = 1.06X10^{-7}M \pm 0.13$$

B) Binding of ANS to BSA

The binding parameters (K_a and n) for ANS were determined from the Scatchard plot (Fig. 5.11a, p.235), as follows :

$$K_a = gradient = \frac{(35.0–20.2)}{(2.3–1.97)} x10^5$$

$$= \frac{14.8}{0.33} x10^5 = 4.49x10^6 M^{-1}$$

and the number of binding sites (n) obtained from the intercept of the line with the r-axis,

i.e. $n = 3.2$

C) Displacement of ANS from BSA by cephalosporins

The data in Table 5.9 (p.234) illustrate the decrease in the fluorescence of ANS in presence of studied cephalosporins. When Scatchard plots were made from the binding data obtained for ANS in the presence of the cephalosporins, almost straight lines were obtained with the initial slopes measured. The intercepts of lines with the abscissa were nearly identical ($n \sim 3$), indicating competition between the probe and the drugs for the same binding sites. The decrease in ANS binding and the subsequent decrease in fluorescence of the probe-protein complex at lower protein concentration (2.76 x 10^{-6} M) was used to calculate the binding constants ($K_{a'}$) of the studied cephalosporins [Table 5.10, p.236], according to equation (5.14).

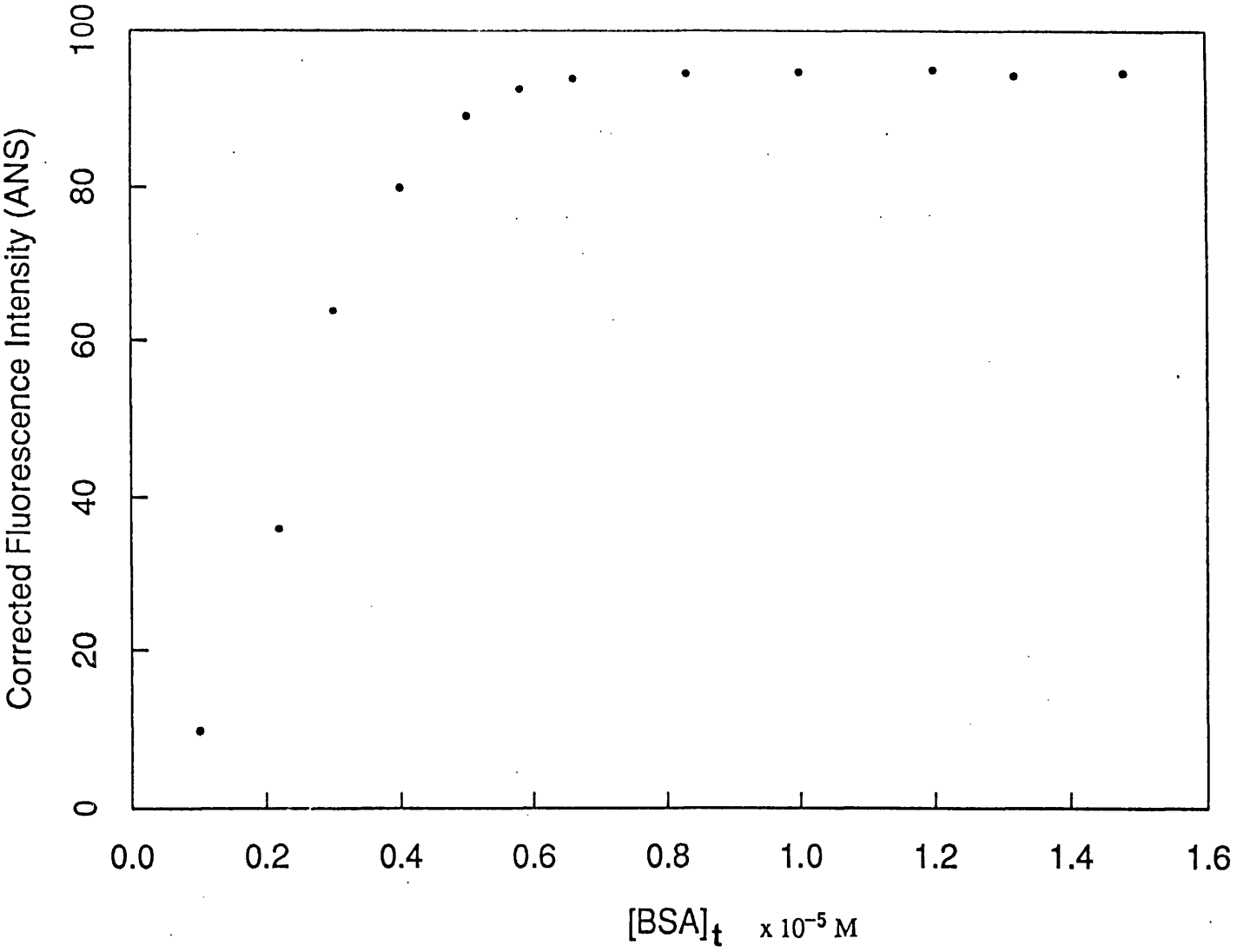


Fig.5.10 : plot of corrected fluorescence inensity of ANS vs $[BSA]_t$, for calculation of Proportionality factor, P.

BSA

Table 5.9 : Corrected fluorescence intensities of ANS in absence and in presence of studied cephalosporins

µl ANS	$[ANS]_t$	Corrected fluorescence intensity (arbitrary unit)					
added[b]	10^{-5}	ANS	CER	CETH	CEZ	CEF	DesCEF
3	0.1	10.15	9.97	9.10	9.20	9.01	9.46
6	0.2	19.10	18.14	16.70	17.40	17.20	19.03
9	0.3	28.25	25.74	23.90	25.10	25.00	26.74
12	0.4	36.50	32.90	30.70	32.60	32.20	34.69
15	0.5	44.20	39.72	37.80	38.90	39.20	41.84
20	0.66	55.65	49.42	43.10	49.50	49.30	52.30
25	0.83	64.80	56.95	54.10	58.00	58.20	59.51
30	1.00	72.10	63.12	62.90	64.00	64.90	67.00
40	1.32	81.10	71.11	73.20	73.50	73.30	75.87
50	1.64	87.10	76.38	79.10	79.60	79.80	82.00
60	1.96	91.10	80.25	84.10	82.60	83.70	86.67
80	2.60	95.00	85.30	88.50	88.20	89.43	93.15
100	3.23	99.00	88.98	92.10	92.20	93.45	95.89
120	3.85	101.00	91.55	95.70	93.60	95.88	97.14

a CER=cefatrizine, CETH=cephalothin, CEZ=cephazolin, CEF=cefotaxime, DesCEF=desacetylcefotaxime, b ANS concentration=1 x 10^{-3}M

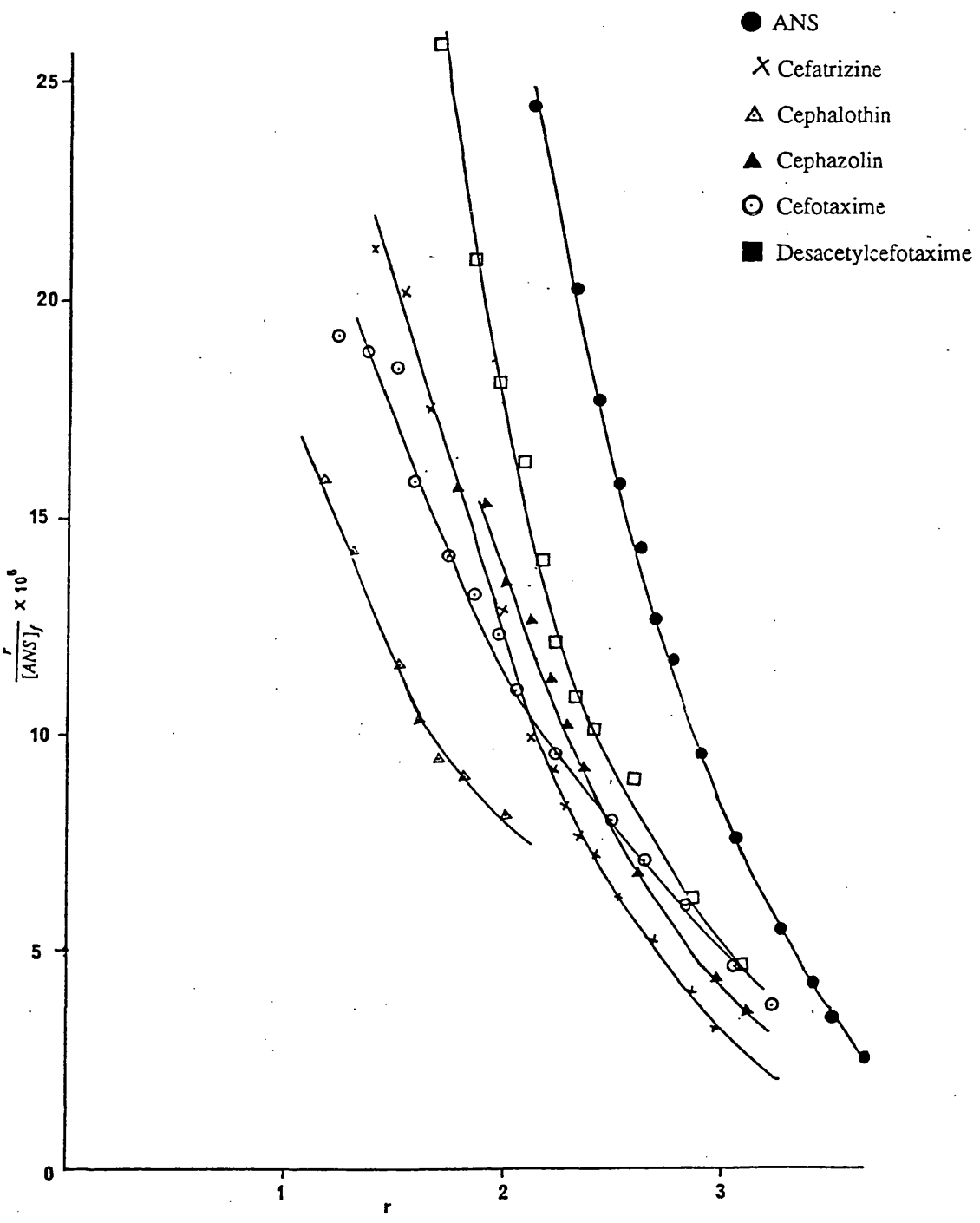


Fig.5.11 : Scatchard plot of ANS for BSA in absence (●) and in presence of the examined cephalosporins.

Table 5.10 : summary of results of the binding data of ANS and the studied cephalosporins for BSA (compared to those of other workers).

Compound	Present work K_a or $K_{a'}$ (M^{-1}) n	Veronese[180]	Kim[185]
ANS	4.50×10^6 **3.2**	0.9×10^6 2.2	2.08×10^6 3.0
Cefatrizine	9.60×10^3 2.8		
Cephalothin	5.18×10^3 2.6	1.2×10^3 2.2	7.0×10^3 3.0
Cephazolin	4.41×10^3 **3.0**		8.19×10^3 3.0
Cefotaxime	4.29×10^3 2.9		$8.98\ 10^3$ 3.0
Desacetyl cefotaxime	2.13×10^3 2.8		

5.5.2 NMR

5.5.2.1 Benzylpenicillin

The proton NMR spectrum of benzylpenicillin in D_2O consists of six groups of peaks arising from : i) a singlet (7.32 ppm, 5H, aromatic), ii) a doublet (5.48 ppm, 1H, 6-H), iii) a doublet (5.40 ppm, 1H, 5-H), iv) a singlet (4.20 ppm, 1H, 3-H), v) an AB double doublet (centered at 3.63 ppm, 2H. $PhCH_2$), and vi) two singlets at 1.55, 1.47 ppm due to the nonequivalent methyl groups.

A typical example of the effect of albumin on the benzylpenicillin spectrum is shown in Fig.5.12 (p.238). The protein appears under of the whole spectrum in a diffuse manner. On addition of albumin, a decrease of T_1 of most protons of the penicillin molecule with increase in protein concentration was observed. Fischer et al.[189] assumed the possibilty that the change in relaxation time is nonspecific rather than an indication of a drug-protein complex, and suggested some control experiments to rule out the nonspecific possibility. Accordingly, we studied the effect of drug-drug interactions on T_1 by using a series of solutions of benzylpenicillin at varying concentrations. The results of measurements of chemical shifts and relaxation rates are recorded in Fig.5.13 (p.239) and Table 5.11 (p.239), respectively.

Significant changes in $\frac{1}{T_1}$ occur with increase in benzyl-penicillin concentration. Chemical shifts do not change by more than 0.1 ppm (5H). Furthermore, the changes in the relaxation rates are paralleled by changes in chemical shifts, i.e., protons of the phenyl group more affected. This is not the case upon adding increasing percentages of BSA to a fixed penicillin concentration, as may be seen from Table 5.12, p.241. Therefore, the assumption that the decrease of T_1 in presence of albumin could be accounted for by interactions between drug molecules can be ruled out.

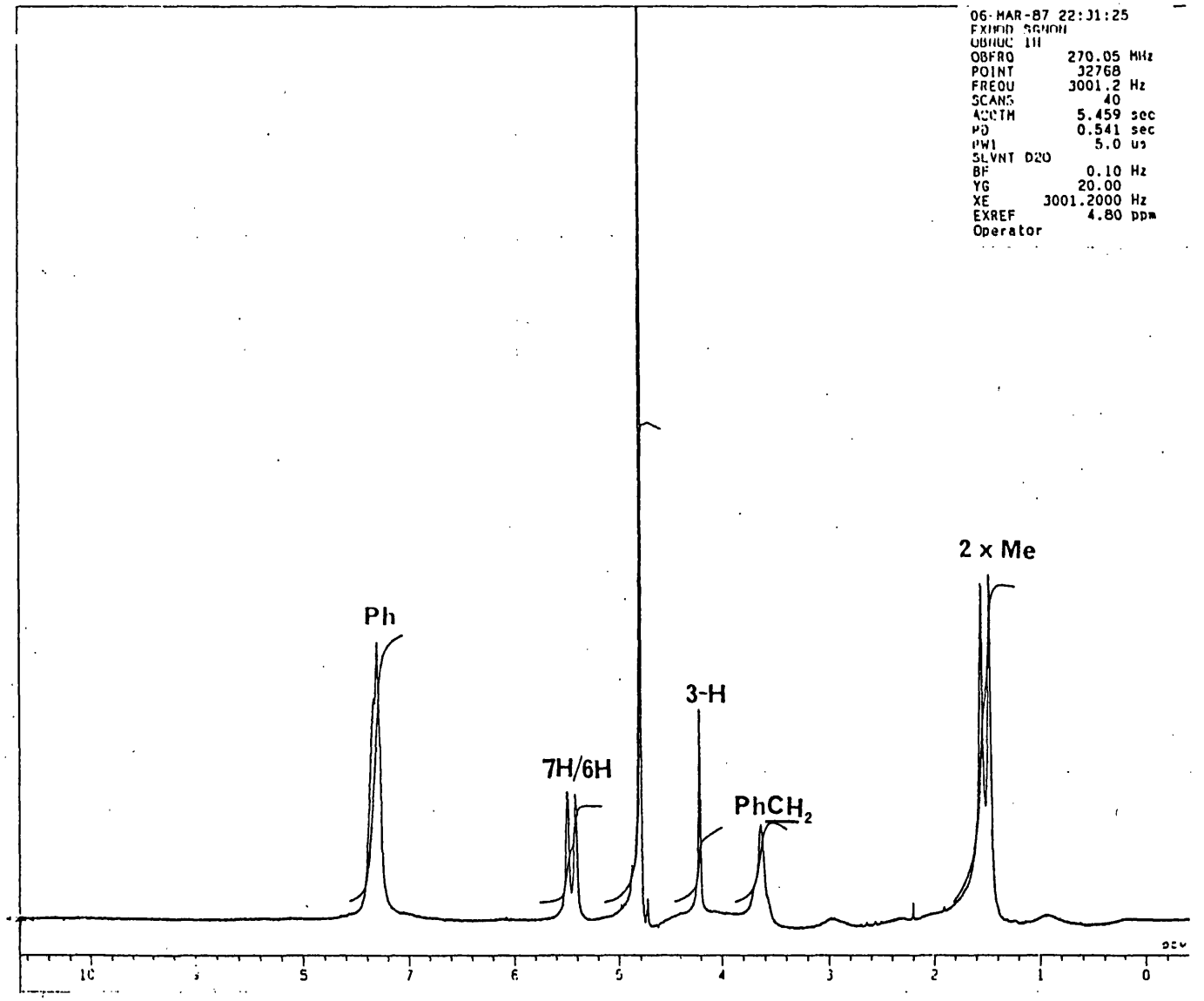


Fig.5.12 : ^{1}H NMR spectrum of benzylpenicillin + 2.5 % BSA.

Table 5.11 : concentration effect on $\frac{1}{T_1}(sec^{-1})$ values of benzylpenicillin

(M)[a]	Me	Me	$ArCH_2$	3-H	5-H	6-H	Ph(5H)
0.1	1.54	1.67	0.85	0.20	0.27	0.23	0.23
0.2	3.03	2.87	1.52	0.47	0.53	0.67	0.37
0.4	3.23	2.94	1.61	0.47	0.52	0.66	0.43
1.0	4.35	4.0	2.33	0.69	0.72	0.89	0.84

a Molar concentration of benzylpenicillin

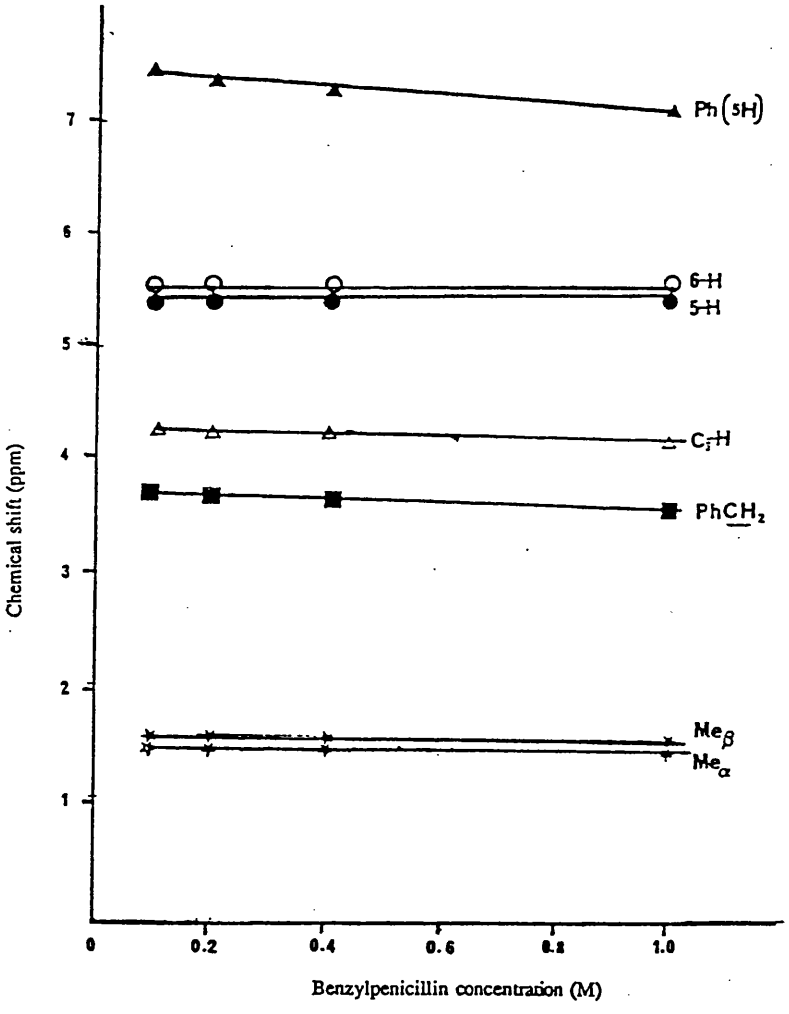


Fig. 5.13 : Effect of concentration on δ ppm values of benzylpenicillin.

Addition of more penicillin to a given penicillin-albumin solution should increase the relaxation rate $\frac{1}{T_1}$ (i.e. decrease T_1), because more penicillin increases the viscosity of the solution, increases the possibility of drug-drug interaction, and certainly should not decrease any intermolecular relaxation effects of the protein on the drug. However, Table 5.13 (p.241) shows a decrease in $\frac{1}{T_1}$ with increase in penicillin concentration, which is exactly opposite to what one expect if the decrease in T_1 were due to viscosity of the solution.

Jardetzky and coworkers[188,189] studied other nonspecific alternatives which might cause broadening of peaks, such as increase in viscosity, effect of using proteins other than albumin on relaxation rates, etc., and all were ruled out as the cause for broadening of peaks.

Since the decrease in T_1 was found to be directly proportional to the total protein concentration (at constant drug concentration), as shown below, therefore it is reasonable to attribute this decrease in T_1 to a specific interaction between drug and protein.

The relaxation times of a series of 0.2 M benzylpenicillin solution in D_2O in presence of the following increasing percentages of BSA were measured: 0, 1.2, 2.5, 3.6, 5.0, 6.6, 8.0, and 10 %. The T_1 and $\frac{1}{T_1}$ data are given in Table 5.14 (p.242). The changes of the chemical shifts were negligible.

5.5.2.2 Cephalosporins

In this section, using a group of cephalosporins with varying binding affinities to serum albumins, the resulting changes in T_1 of the resonance signals arising from the various hydrogen-containing parts of the molecule, were measured.

Addition of more penicillin to a given penicillin-albumin solution should increase the relaxation rate $\frac{1}{T_1}$ (i.e. decrease T_1), because more penicillin increases the viscosity of the solution, increases the possibility of drug-drug interaction, and certainly should not decrease any intermolecular relaxation effects of the protein on the drug. However, Table 5.13 (p.241) shows a decrease in $\frac{1}{T_1}$ with increase in penicillin concentration, which is exactly opposite to what one expect if the decrease in T_1 were due to viscosity of the solution.

Jardetzky and coworkers[188,189] studied other nonspecific alternatives which might cause broadening of peaks, such as increase in viscosity, effect of using proteins other than albumin on relaxation rates, etc., and all were ruled out as the cause for broadening of peaks.

Since the decrease in T_1 was found to be directly proportional to the total protein concentration (at constant drug concentration), as shown below, therefore it is reasonable to attribute this decrease in T_1 to a specific interaction between drug and protein.

The relaxation times of a series of 0.2 M benzylpenicillin solution in D_2O in presence of the following increasing percentages of BSA were measured: 0, 1.2, 2.5, 3.6, 5.0. 6.6, 8.0, and 10 %. The T_1 and $\frac{1}{T_1}$ data are given in Table 5.14 (p.242). The changes of the chemical shifts were negligible.

5.5.2.2 Cephalosporins

In this section, using a group of cephalosporins with varying binding affinities to serum albumins, the resulting changes in T_1 of the resonance signals arising from the various hydrogen-containing parts of the molecule, were measured.

Table 5.12 : Chemical shifts (ppm) of 0.2 M benzyl penicillin-BSA soluions

BSA %	Me	Me	$ArCH_2$	3-H	5-H	6-H	Ph(5H)
0.0	1.48	1.56	3.64	4.22	5.40	5.49	7.33
1.2	1.47	1.56	3.63	4.21	5.41	5.48	7.32
2.5	1.48	1.56	3.64	4.22	5.42	5.49	7.33
3.6	1.47	1.55	3.63	4.21	5.42	5.48	7.33
5.0	1.47	1.55	3.63	4.21	5.41	5.48	7.32
6.6	1.46	1.54	3.63	4.20	5.40	5.47	7.31
8.0	1.46	1.54	3.62	4.20	5.40	5.47	7.31
10.0	1.47	1.55	3.63	4.20	5.41	5.48	7.32

Table 5.13 : T_1 (sec) and $\frac{1}{T_1}(sec^{-1})$ of benzylpenicillin signals as a function of drug concentration at constant BSA concentration (2.5%) in D_2O

(M)[a]	Me	Me	$ArCH_2$	3-H	5-H	6-H	Ph(5H)
0.2							
T_1	0.33	0.35	0.70	1.19	1.37	1.23	1.50
$\frac{1}{T_1}$	3.06	2.86	1.41	0.84	0.73	0.81	0.67
0.4							
T_1	0.37	0.40	0.62	1.77	1.90	1.73	1.61
$\frac{1}{T_1}$	2.67	2.51	1.62	0.56	0.53	0.58	0.62

a Molar concentration of benzylpenicillin

Table 5.14 : T_1 (sec) and $\frac{1}{T_1}(sec^{-1})$ of solutions of 0.2M benzylpenicillin + BSA in D_2O :

BSA %	Meα	Meβ	$ArCH_2$	3-H	5-H	6-H	Ph(5H)
	T_1	T_1	T_1	T_1	T_1	T_1	T_1
	$(\frac{1}{T_1})$	$(\frac{1}{T_1})$	$(\frac{1}{T_1})$	$(\frac{1}{T_1})$	$(\frac{1}{T_1})$	$(\frac{1}{T_1})$	$(\frac{1}{T_1}$
0.0	0.33	0.35	0.66	2.14	1.89	0.53	1.50
	(3.03)	(2.86)	(1.52)	(0.47)	(0.53)	(0.67)	(0.37)
1.2	0.32	0.35	0.72	1.36	1.44	1.29	1.66
	(3.13)	(2.86)	(1.39)	(0.74)	(0.69)	(0.78)	(0.60)
2.5	0.32	0.35	0.72	1.16	1.33	1.19	1.45
	(3.13)	(2.86)	(1.39)	(0.86)	(0.75)	(0.84)	(0.69)
3.6	0.40	0.43	0.77	1.10	1.32	1.10	1.34
	(2.50)	(2.33)	(1.30)	(0.91)	(0.76)	(0.91)	(0.75)
5.0	0.49	0.47	0.79	1.06	1.24	1.13	1.26
	(2.04)	(2.13)	(1.27)	(0.94)	(0.81)	(0.88)	(0.79)
6.6	0.43	0.47	0.74	0.79	1.04	0.96	1.07
	(2.33)	(2.13)	(1.35)	(1.27)	(0.96)	(1.04)	(0.93)
8.0	0.42	0.46	0.75	0.84	1.04	0.92	1.02
	(2.38)	(2.17)	(1.33)	(1.19)	(0.96)	(1.09)	(0.98)
10.0	0.48	0.50	0.76	0.81	1.01	0.93	1.00
	(2.08)	(2.00)	(1.32)	(1.23)	(0.99)	(1.08)	(1.00)

According to the relative extent of change in T_1, the major part of the drug molecule involved in binding to the serum albumin can be identified.

In this study, the cephalosporins chosen were classified according to their binding affinities (see results reported in section 5.5.1) into :

a) strongly binding : cephalothin, cephazolin;

b) moderate affinity : cefotaxime, cephaloridine;

c) weak affinity : cephalexin, cefadroxil.

The NMR chemical shifts and assignments of the protons of the studied cephalosporins were identified and discussed in chapter three.

Small changes (0.03 - 0.05 ppm) in chemical shifts of all spectra of each cephalosporin (0.1 M) in the presence of several concentrations of serum albumin were observed [Table 5.15, pp.244-245]. These might result from error in referencing (HOD peak at 4.80 ppm) as the changes were not uniform, i.e., with increasing protein concentration the peaks sometimes appeared upfield and in other cases lowfield. (Therefore, the study of drug-protein interactions cannot be carried out on the basis of the changes in chemical shifts).

The effects of BSA (or HSA) concentration on the relaxation rates of studied cephalosporins are summarized in Figs. 5.14 - 5.17 (pp.246-247) as plots of $\frac{1}{T_1}$ for each proton versus the concentration of protein. Table 5.16 (p.248) reports the $\frac{T_1 free}{T_1 bound}$ ratios for each cephalosporin and for all observable protons.

Table 5.15 : Effect of serum albumin on chemical shifts (δ ppm) of 0.1 M cephalosporins (with reference to HDO peak at 4.80 ppm)

a) Cephalothin

Conc. of S.A.%	2-CH_2 α	β	3-CH_2 α	β	6-H	7-H	CH_3	PhCH2	Aromatics 1	2
0.0	3.34	3.61	4.71	4.87	5.08	5.64	2.10	3.90	7.04	7.36
0.5	3.34	3.60	4.70	4.87	5.08	5.62	2.09	3.88	7.03	7.36
1.0	3.34	3.61	4.70	4.87	5.08	5.62	2.09	3.88	7.03	7.36
1.5	3.35	3.61	4.71	4.88	5.09	5.64	2.10	3.90	7.03	7.36
2.0	3.35	3.62	4.71	4.88	5.10	5.64	2.10	3.90	7.04	7.36
2.5	3.34	3.60	4.74	b.p.	5.10	5.64	2.11	3.90	7.04	7.37
5.0	b.p.	b.p.	4.75	b.p.	5.11	5.65	2.12	3.91	7.05	7.37
10.0	b.p.	b.p.	b.p.	b.p.	b.p.	5.68	2.12	3.92	7.06	7.37

b) Cephazolin

0.0	3.42	3.79	3.92	4.50	5.07	5.65	2.71	5.52	9.28	
0.5	3.40	3.77	3.91	4.48	5.06	5.64	2.70	5.51	9.25	
1.0	3.40	3.77	3.91	4.48	5.06	5.64	2.70	5.51	9.25	
1.5	3.40	3.77	3.91	4.48	5.06	5.64	2.69	5.51	9.26	
2.5	3.42	3.79	3.93	4.51	5.09	5.66	2.71	5.54	9.27	
5.0	b.p.	b.p.	b.p.	b.p.	5.10	5.66	2.71	5.54	9.28	

c) Cephaloridine

Conc. of S.A.%	2-CH_2 α	β	3-CH_2 α	β	6-H	7-H	$PhCH_2$	Thi.H 1	2	Pyr.H 1	2	3
0.0	3.14	3.56	5.32	5.52	5.12	5.64	3.86	6.99	7.32	8.08	8.55	8.91
0.5	3.15	3.58	5.33	5.53	5.14	5.65	3.88	7.00	7.32	8.08	8.55	8.94
1.0	3.15	3.57	5.33	5.53	5.13	5.65	3.87	6.99	7.33	8.07	8.56	8.93
1.5	3.14	3.56	5.32	5.52	5.15	5.67	3.89	7.01	7.33	8.10	8.58	8.95
2.5	3.17	3.58	5.36	5.53	5.13	5.67	3.88	7.01	7.33	8.10	8.57	8.94
5.0	3.18	3.54	5.36	5.50	5.13	5.65	3.86	6.98	7.31	8.07	8.56	8.92

Table 5.15 **cont.**

d) Cefadroxil

Conc. of S.A.%	2-CH_2 α	2-CH_2 β	3-CH_3	6-H	7-H	PhCH
0.0	3.21	3.49	2.09	4.99	5.73	4.95
1.2	3.21	3.48	2.08	4.99	4.73	4.95
2.5	3.21	3.49	2.08	4.99	5.73	4.95
5.0	b.p.	b.p.	2.04	b.p.	5.71	b.p.
6.6	b.p.	b.p.	2.02	b.p.	5.70	b.p.

e) Cephalexin

0.0	3.17	3.46	2.07	5.05	5.65	5.24
0.5	3.17	3.46	2.07	5.05	5.65	5.24
1.0	3.15	3.46	2.05	5.04	5.65	5.23
1.5	3.14	3.43	2.04	5.02	5.62	5.22
2.0	3.11	3.45	1.97	5.03	5.65	5.23
2.5	3.15	3.48	2.03	5.06	5.66	5.26
5.0	3.12	3.46	1.97	5.06	5.67	5.24

f) Cefotaxime

Conc. of S.A.%	2-CH_2 α	2-CH_2 β	3-CH_2 α	3-CH_2 β	3'-CH_3	NOMe	6-H	7-H	Ph.H
0.0	3.39	3.67	4.70	4.87	2.08	3.97	5.20	5.81	6.99
1.0	3.41	3.69	4.72	4.89	2.10	3.99	5.22	5.83	7.02
1.8	3.40	3.68	4.71	4.87	2.08	3.98	5.21	5.81	7.00
2.6	3.39	3.67	4.73	4.88	2.08	3.97	5.20	5.81	6.98
3.6	3.39	3.67	b.p.	b.p.	2.07	3.96	5.20	5.80	6.98
4.7	3.39	3.67	b.p.	b.p.	2.07	3.96	5.19	5.80	6.98
5.7	3.39	3.67	b.p.	b.p.	2.06	3.96	5.19	5.79	6.98
7.0	3.39	3.67	b.p.	b.p.	2.06	3.95	5.18	5.79	6.97

g) Cefotaxime lactone

Conc. of S.A.%	2-CH_2 α	2-CH_2 β	lactone CH_2 α	lactone CH_2 β	NOMe	6-H	7-H	Ph.H
0.0	3.75	3.93	5.12		4.08	5.31	5.94	7.14
0.5	3.75	3.93	5.12		4.08	5.31	5.94	7.14
1.0	3.75	3.93	5.12		4.08	5.32	5.95	7.14
1.5	3.75	3.93	5.12		4.08	5.31	5.94	7.14
2.0	3.76	3.94	5.13		4.09	5.32	5.95	7.14
2.5	3.76	3.94	5.13		4.08	5.31	5.95	7.14

a Conc. of S.A.%=concentration of serum albumin in %, b.p.=broad peak, Thi.H=thienyl protons, Pyr.H=pyridyl protons, Ph.H=phenyl protons.

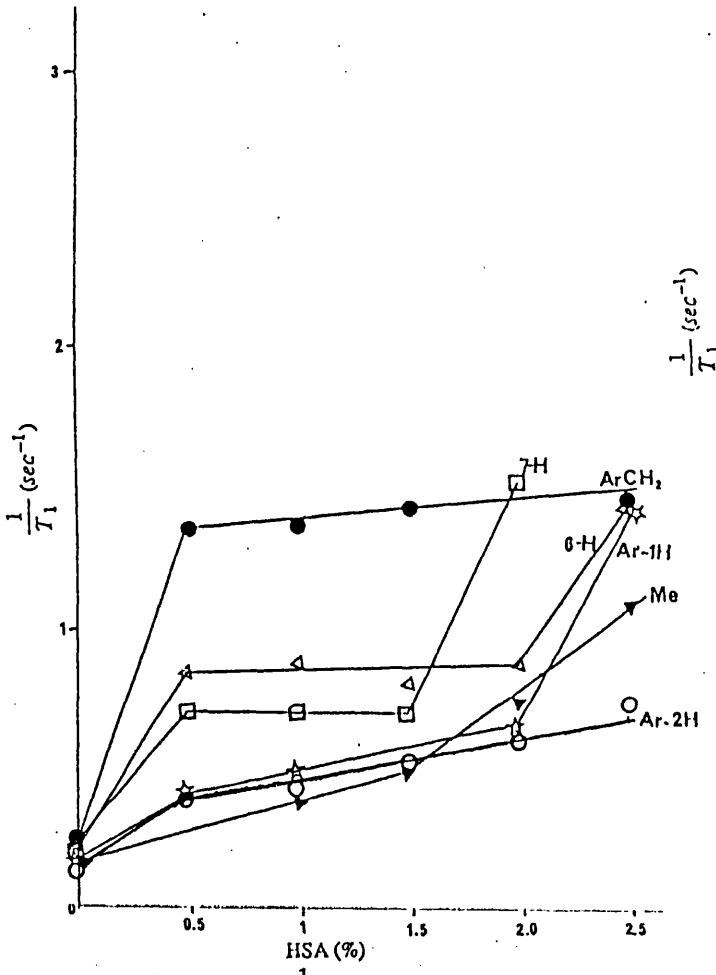


Fig.5.14 : Changes in $\frac{1}{T_1}$ of 0.1 M cephalothin protons as function of HSA concentration (%)

(N.B. Other protons show insignificant $\frac{1}{T_1}$ changes).

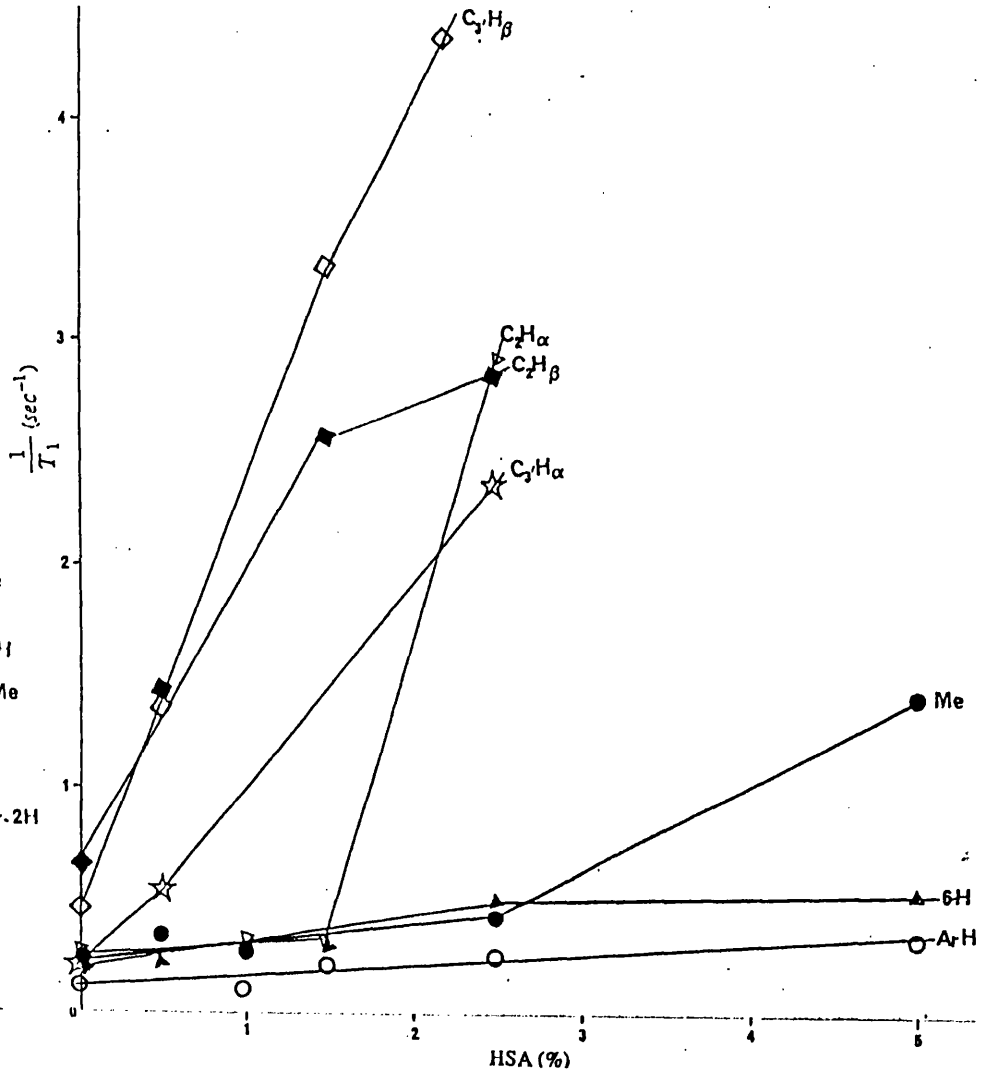


Fig.5.15 : Changes in $\frac{1}{T_1}$ of 0.1 M cephazolin protons as function of HSA concentration (%)

(N.B. Other protons show insignificant $\frac{1}{T_1}$ changes).

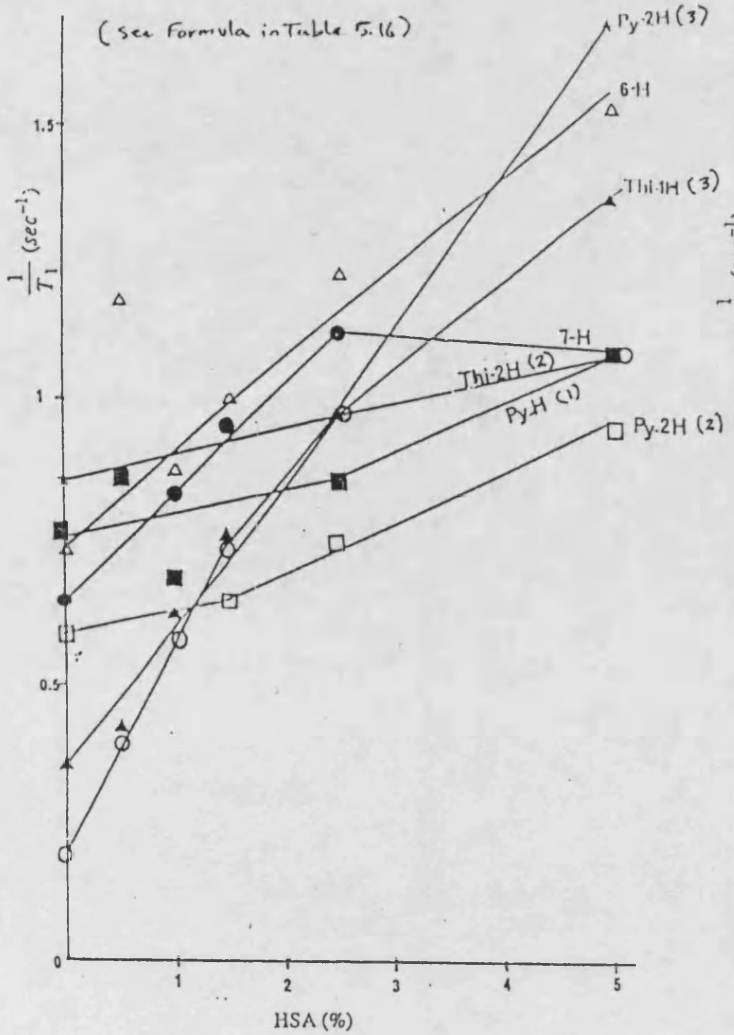


Fig.5.16 : Changes in $\frac{1}{T_1}$ of 0.1 M cephaloridine protons as function of HSA concentration (%)

Thi=thienyl, Py=Pyridyl

(N.B. Other protons show insignificant $\frac{1}{T_1}$ changes).

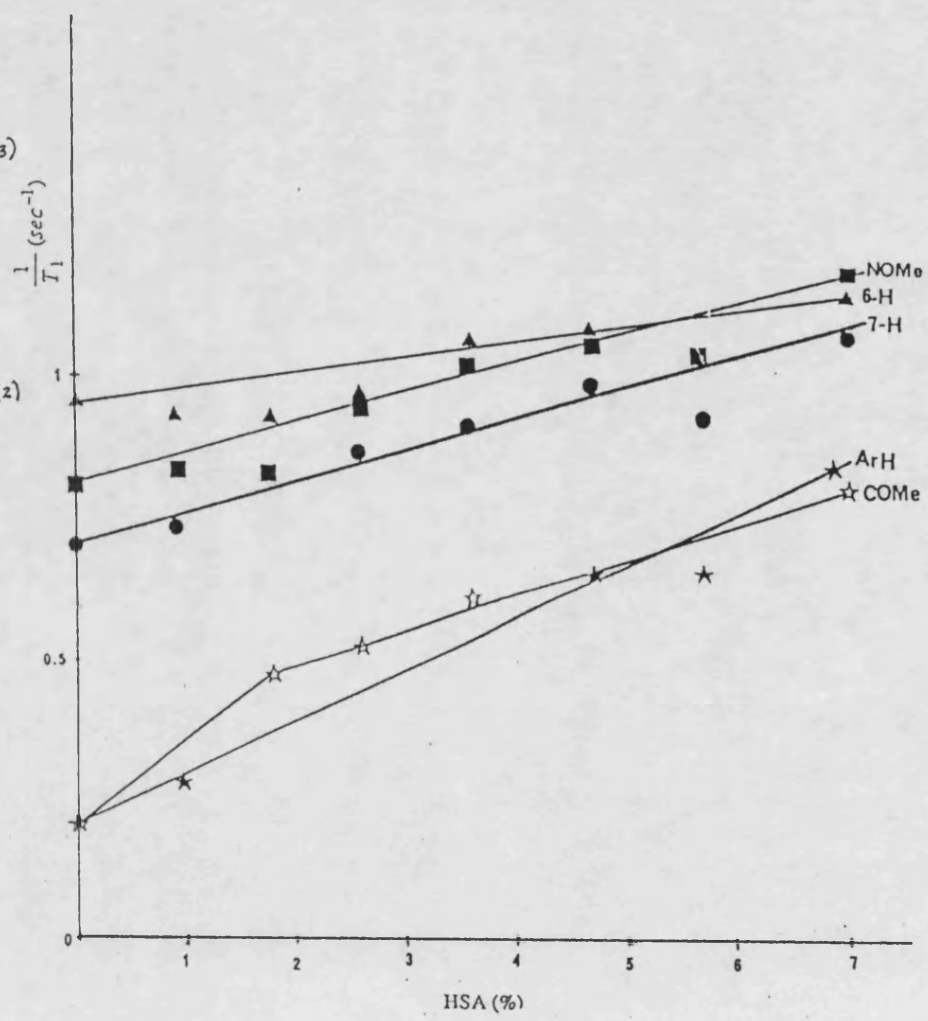
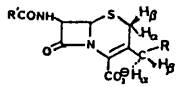


Fig.5.17 : Changes in $\frac{1}{T_1}$ of 0.1 M cefotaxime protons as function of HSA concentration (%)

(N.B. Other protons show insignificant $\frac{1}{T_1}$ changes).



	2-CH_2		3'-CH_2		6-H	7-H	R			R'			Notes
	α	β	α	β			1	2	3	1	2	3	
Cefalexin $1/T_{1f}$	2.13	2.44			0.47	0.35	1.22			0.40	0.49		HSA
R = H $1/T_{1b}$	0.67	0.69			0.52	0.55	0.64			0.55	0.57		
R' = T_{1f}/T_{1b}	0.31	0.28			1.11	1.57	0.52			1.38	1.16		
Cefadroxil $1/T_{1f}$	2.56	2.70			0.45	0.67	1.52			1.19	0.69	11.05	a: T_1 affected by solvent peak, giving very long T_1
R = H $1/T_b$	2.44	2.63			0.62	0.75	1.45			(a)	0.73	1.09	
R' = T_{1f}/T_{1b}	0.88	0.87			1.08	1.13	0.88				1.08	1.04	HSA
Cefotaxime Na $1/T_{1f}$	3.57	3.45	22.44	2.38	0.96	0.71	0.86			1.11	0.20		b: peaks obscured by HOD peak at 6.53
R = $-O-CO-CH_3$ $1/T_{1b}$	2.38	1.39	2.22	(b)	0.97	0.87	0.85			0.96			
R' = T_{1f}/T_{1b}	0.67	0.40	0.91		1.01	1.23	0.99			0.86	2.65		BSA
Cefaloridine $1/T_{1f}$	2.70	3.57	3.33	3.03	0.75	0.64	0.76	0.59	0.86	1.45	0.35	0.19	
R = $1/T_{1b}$	1.69	1.67	1.69	11.96	1.23	1.12	0.86	0.75	0.97	0.71	0.97	0.97	HSA
R' = T_{1f}/T_{1b}	0.63	0.47	0.51	0.65	1.64	1.75	1.13	1.27	1.13	0.49	2.77	5.11	
Cephalothin Na $1/T_{1f}$	0.32	0.26	0.27	0.20	0.20	0.24	0.17			0.23	0.17	0.15	c: not teletyped, due to broadness of peak.
R = $-O-CO-CH_3$ $1/T_{1b}$	2.56	2.56	1.92	(c)	1.27	1.06	1.11			1.45	1.44	0.74	
R' = T_{1f}/T_{1b}	8.0	9.85	7.11		6.35	4.45	6.53			6.30	8.47	4.93	HSA
Cefazolin Na $1/T_{1f}$	0.33	0.64	0.22	0.48	0.20	0.30	0.29			0.21	0.12		
R = $1/T_{1b}$	2.86	2.86	2.63	5.10	0.45	0.52	0.42			0.51	0.26		HSA
R' = T_{1f}/T_{1b}	8.67	4.47	11.95	10.63	2.25	1.73	1.45			2.43	2.17		
Cefotaxime lactone			$NOCH_3$	Lactone									
R = $1/T_{1f}$	0.11	2.5	0.07	2.44	0.09	0.11	0.11						
$1/T_{1b}$	2.56	2.63	0.30	3.57	0.30	0.39	0.12						
R' = T_{1f}/T_{1b}	23.27	1.05	4.29	1.46	3.33	3.55	1.05						

Table
Fig. 5.16: Free and bound relaxation times (T_1) and $T_{1f_{free}}/T_1$ bound ratios for each measurable proton signal of cephalosporins (0.1M) in the presence of 2.5 % serum albumin. (f: free, b: bound).

5.6 Discussion

5.6.1 Discussion of fluorimetry results

Fig. 5.7 (p.225) shows the dramatic change in the ANS fluorescence upon addition of HSA. It was reported[216,217] that the binding species is the anion rather than the neutral molecule of ANS. Proteins such as serum albumin are known to bind strongly with small organic molecules with anionic groups[218]. Therefore, in case of ANS, the acid group might interact with positively charged residues in the protein to form complexes, with the binding reinforced by the hydrophobic interactions between the protein and ANS. The most strongly hydrophobic sites on the protein were probably primarily involved in the binding. The fluorescence change of the probe when bound to serum albumin could be due to change in the charge of protein molecules[219]. The hydrophobic nature of the binding site can account for major part of the blue shift fluorescence exhibited by ANS[217].

The enhancement in fluorescence intensity of ANS was approximately linear at high concentration of serum albumin, 1.0×10^{-5} M (Fig.5.11, p.235), suggesting a proportional increase in the bound form of ANS in the system. The curve of low concentration of serum albumin, 1.0×10^{-6} M (Fig. 5.9, p.230), might indicate that the ANS was only partially bound (due to saturation of the binding sites of the protein).

Fig. 5.9 as well as Table 5.6 (p.228) show the reduction in the fluorescence intensities of ANS-serum albumin complex upon addition of cephalosporins, as indication of a reversible competition between ANS and drug for the same binding sites. If the competition was not reversible, the affinity of ANS would remain the same, but the total number of binding sites would have been greatly reduced[220]. In our case the total number of binding sites remained the same and competition resulted in a reduced affinity of the ANS for serum albumin, indicating the reversibility of competition.

Based on the Scatchard plots (Fig. 5.9 and Fig. 5.11), ANS appeared to have one primary binding site on HSA and 3 primary sites on the BSA molecule under the described

experimental conditions. The intercepts of the Scatchard plots obtained for ANS in the presence of cephalosporins occur at the same point, therefore indicating binding at the same or adjacent sites.

Comparison of the HSA and BSA fluorimetry results (Tables 5.8, p.231. and 5.10, p.236) showed minor differences in the binding constants of ANS for HSA(K_a = 3.36 x 10^{-6}M) and that for BSA (K_a = 4.50 x 10^{-6}M). As for the affinity constants of cephalosporins, only small variations occurred with HSA and BSA, but in each case a similar value was observed for the studied antibiotics, that is a stronger binding for cephalothin, and relatively weaker bindings for cephazolin and cefotaxime.

The stability of a drug-protein complex is expressed by its binding constant, which is also important for the pharmacokinetic behaviour of the drug[221]. Accordingly, cephalothin, cefatrizine and cephazolin can be classified as highly bound, with affinities higher than 4.0 X 10^3 M^{-1}, cefotaxime can be classified as moderately bound, cefalexin, with affinity below 2.0 X 10^3 M^{-1}, as weakly bound.

Results of binding constants of cefotaxime (~ 4.0 X $10^3 M^{-1}$), desacetylcefotaxime (~ 2.0 X $10^3 M^{-1}$) and cefotaxime lactone (10 X $10^3 M^{-1}$) indicate that the degree of binding to serum albumin probably depends on the overall polarity of the molecule, i.e., the increased polarity of the molecule may reduce the binding affinity.

5.6.2 Discussion of NMR results

The formation of intermolecular complexes can be detected by observing the accompanying changes in either the relaxation time or the chemical shifts or both.

5.6.2.1 Benzylpenicillin

It appears, from the benzylpenicillin-albumin relaxation measurement results (section 5.5.2.1) there is indeed a significant decrease in T_1 values, greater for the phenyl and C-3

protons, than either the proton on C-6 or C-5. These changes in the relaxation times of the peaks concerned reflects a restriction in their rotational freedom as the molecule binds to the protein[187]. Fig.5.18 (p.252) shows that the relative decrease in the relaxation time of benzylpenicillin (0.2 M) is found to be a function of protein concentration, although this is not strictly linear. The observed relaxation rate increases more from 0 to 1.2 % than from 1.2 to 10 % albumin concentration, especially in cases of aromatic proton and the proton of the carbon 3. This latter finding may indicate the dependence of relaxation on the penicillin : albumin ratio. Below 1.8×10^{-4}M (1.2%) BSA concentration the ratio is greater than 1000, i.e., at 1.8×10^{-4} BSA (where the slope breaks-down), the number of penicillin molecules is about 1000 times higher than that of albumin molecules, which means that the latter should be saturated with penicillin, with the consequence that the bound ligand increases rapidly : $\frac{1}{T_1}$ is the representative relaxation rate from $\frac{1}{T_1}$ free to $\frac{1}{T_1}$ bound with a linear effect from 0 to 1.8×10^{-4} (0 to 1.2 %) BSA concentration. In cases of concentrations greater than 1.8×10^{-4} (>1.2 %) the penicillin binding to the saturated albumin decreases inducing a fall in slope, as shown in Fig.5.18.

As mentioned above, the decrease in T_1 values of the phenyl and C-3 protons was greater than those of the other protons in the penicillin molecule. This result might indicate that these protons were involved in the formation of penicillin-protein complex, while other portions of the molecule retained freedom of motion to a greater extent. As the phenyl group is a very hydrophobic structure, this suggests the hydrophobic nature of binding of penicillin (via aromatic protons) to BSA. The broadening of the proton signal attached to carbon-3 can be explained as follows : BSA contains 16 histidine residues per molecule[158], which are positively charged in the range pH 6 - 7[222]. Therefore, the possibilty exist for an ionic interaction between the positively charged histidine and the carboxyl group (at C-3) of the penicillin, affecting the T_1 of the β proton at C-3. This might be suggested as another site of penicillin-protein interaction.

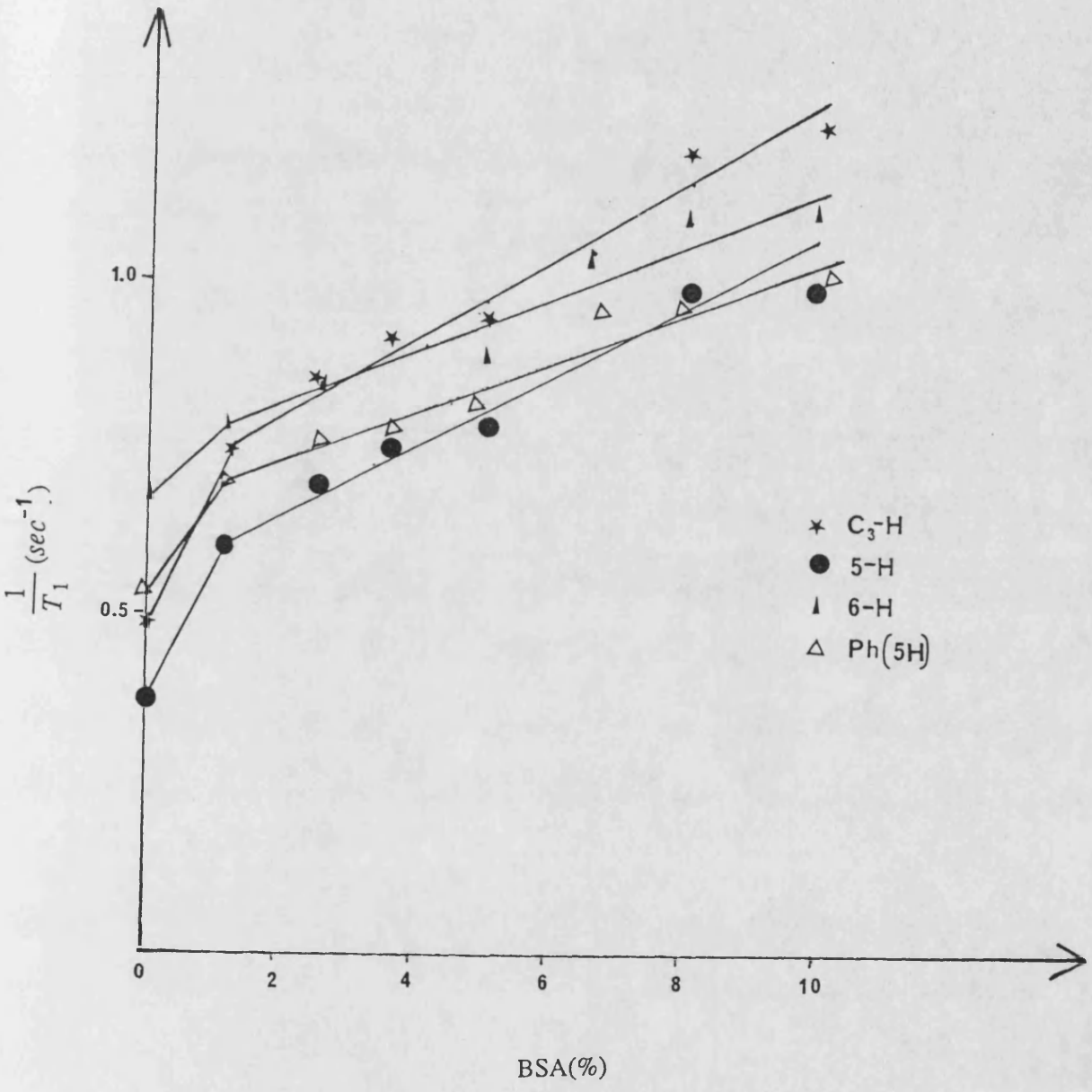


Fig.5.18 : Changes in relaxation rates ($\frac{1}{T_1}$) of the phenyl protons and those of C_3, C_5 and C_6 of benzylpenicillin (0.2M) as a function of BSA concentration.

5.6.2.2 Discussion of cephalosporins NMR results

In the analysis of NMR spectra of cephalosporins in presence of serum albumin, the chemical shifts showed negligible changes [Table 5.15, pp.244-245], while relaxation times (T_1) showed variable changes depending on the nature of the proton-containing groups in the cephalosporin molecule (Figs.5.14-5.17, pp.246-247). Those which were preferentially stabilised by the formation of the complex were affected to a greater extent than those of groups whose rotational freedom was not restricted[223,224]. A corresponding decrease of the relaxation times was observed [Table 5.16, p.248].

The insignificant changes in the chemical shifts of the free and bound molecules of the studied cephalosporins [see Table 5.15] indicate a rapid exchange between the two states supported by the shape of the spectra and our fluorimetric results which indicate that the cephalosporins exchange rapidly with at least one site on HSA, and three sites on BSA.

As shown under the discussion of penicillin-albumin interaction, the formation of intermolecular complexes can be detected by observing the accompanying changes in the relaxation time. The analysis of NMR spectra helped to assess which part of the molecule participate in drug-protein interactions[158,164].

As the $\frac{T_1 free}{T_1 bound}$ ratio increases especially for protons that get close to the serum albumin as a consequence of stronger interactions, therefore, it is logical to investigate the drug-binding sites as well as extent of binding by comparing those T_1 proton ratios.

The results presented in Table 5.16 showed that the relaxation rates ($\frac{1}{T_1}$) of certain proton-containing groups were increased by approximately two to tenfold, depending on strength of binding, in the presence of 2.5% serum albumin. Accordingly, the results in Table 5.16 indicate that, cephalothin and cephazolin were strongly bound to HSA (as they showed larger changing increments in the relaxation rates). Both drugs showed two classes

of binding sites for serum albumin, one with very strong affinity near 2-CH_2 and 3'-CH_2 (showing a changing factor of 7 to 12) and the other site showing a lesser binding affinity near the R_3 group (with a $\frac{T_1 free}{T_1 bound}$ ratio less than 7).

In case of cephalothin, the actual relaxation rate measurements (in sec^{-1}) showed the following values : in D_2O a) 2-CH_2 = 0.32, 0.26; b) 3'–CH_2 = 0.27, 0.20; c) R_3 group, 0.23, 0.17. 0.15; + HSA a) 2-CH_2 = 2.86, 2.56; b) 3'-CH_2 = 1.92; c) R_3 = 1.45, 1.44, 0.74.

Cephazolin showed the following values : in D_2O a) 2-CH_2 0.33, 0.64; b) 3'-CH_2 0.22, 0.48; c) R_3 0.21, 0.12; + HSA a) 2-CH_2 2.86; b) 3'-CH_2 2.63, 5.10; and c) R_3 0.51, 0.26 sec^{-1}.

With cephaloridine, which showed relatively strong binding but less than cephalothin and cephazolin, it seems that the increased polarity of the 3-substituent ($C_5 H_5 N^+$) is the cause of the reduced binding affinity of the molecule as a whole, and in particular, those of 2-CH_2 and 3'-CH_2 [see Table 5.16]. The primary binding site appeared to be the theinyl moeity, showing the largest changing factor compared to the other parts of the molecule, supported by the following $\frac{1}{T_1}$ measurement values : in D_2O 0.35. 0.19 sec^{-1}; + HSA 0.97, 0.97 sec^{-1}

Cefotaxime showed moderate affinity to serum albumin, with the aromatic moeity as the primary binding site, with a changing factor of 2.5 - 5.1 compared to that of the above-mentioned compounds, and to a lesser extent near the lactam ring protons, with a ratio of 1.2-1.8 [see Table 5.16]. The presence of the $NOCH_3$ group in the 7-substituent might affected the binding of the drug to albumin.

On the other hand. drugs like cephalexin and cefadroxil, which are known to bind very weakly, showed low $\frac{T_1 free}{T_1 bound}$ ratios (<2.0) whatever proton is considered. It seems that the amino substitution might dramatically reduced the binding of these compounds to albumin as clearly shown by the data of Table 5.16, pp.248.

Furthermore, Figs.5.14 - 5.17 (pp.246-247) show that the relaxation rates of the chosen peaks for the particular cephalosporin increased with the addition of serum albumin. The extent of change in $\frac{1}{T_1}$ of some of these peaks showed dependence on the drug : albumin ratio, most of which showed rapid linear increase in $\frac{1}{T_1}$ at low albumin concentration to a point where the slope breaks-down to a slow increase in $\frac{1}{T_1}$ (see Figs.5.14 - 5.16); the latter stage might indicate some saturation of the binding sites on albumin. Fig.5.15 (p.246) showed another rapid increase on $\frac{1}{T_1}$ with HSA concentration, after the saturation stage, which may indicate availability of new binding sites of same characters as the previous. This might be explained as that the protein undergo an unfolding process in case of cephazolin, such that further sites become accessable to the drug.

In summary, from the above discussion and data of Table 5.16, it seems that the serum albumin binding is dependent upon the interaction between the 7-acyl group and the 3-methyl substituent. In the group of sites of high affinity, the methylthienyl, methylthiadiazole or methyltetrazole nuclei would probably bind to serum albumin by means of electrostatic forces. In the case of cefotaxime, the presence of $NOCH_3$ group in the 7-acyl substituent may reduce extent of binding. The presence of an additional substituent like NH_2 in the cefalexin molecule is characteristic of weak binding.

5.6.3 Comparison of fluorimetry and NMR results

It is worthmentioning that one of the purposes of the present study was to examine the potentials of fluorescence probe and T_1 ^1H NMR techniques in the area of cephalosporin-protein interactions. Both methods were chosen because of the following reasons :

1. Their sensitivity to changes upon binding.

2. Only microquantities of material required.

3. Direct evaluation of the binding properties of cephalosporins.

4. The two methods complement each other.

5. Availability of advanced instrumentation.

The fluorimetry method was used to calculate the binding parameters (affinity constant of binding and site number) of the chosen cephalosporins to bovine and human serum albumin, according to Scatchard's method. The fluorescence probe (ANS) was employed as a reporter group molecule for fluorescence measurements in order to detect binding at the hydrophobic sites of serum albumin. ANS was also used for the determination of the binding ability of cephalosporins to albumin.

The high-resolution ^1H NMR results provided information about the protons of the drug molecules which were involved in the binding to serum albumin, on the basis of changes of the longitudinal relaxation times ($\frac{1}{T_1}$) of protonic signals. Furthermore, the technique showed the extent to which various functional groups on the drug molecule participate in drug-protein interactions, by utilizing the extreme sensitivity of $\frac{1}{T_1}$ relaxation rates to small variations in the molecular environment of a proton species to show clearly how measurement of these parameters (T_1 and $\frac{1}{T_1}$) reveals formation of intermolecular

complexes.

When comparing results of the two techniques in terms of affinity of cephalosporins towards albumin, both showed same order of rank; e.g. cephalexin showed very weak binding to albumin (K_a about 1.7 X $10^3 M^{-1}$), while the NMR method revealed no significant changes in the relaxation times of the protons of the drug molecule on addition of albumin [Table 5.16]. Therefore, the two techniques can complement each other, and combined provide valuable information for elucidating the nature of binding of cephalosporins and serum albumin.

In the present study, the major disadvantage of the fluorimetric technique is the lengthy procedures needed for the quantification of results; that of NMR is the time factor as it involved the use of series of albumin concentrations, each needed at least six hours to obtain the required T_1 spectrum.

5.6.4 Comparison with other methods from literature

Many techniques, other than fluorimetry and NMR, have been used for studies of binding of cephalosporins to serum albumin, which have generally yielded somewhat limited information on the complex formation. For instance, the use of equilibrium dialysis and ultrafiltration in deriving structural information regarding the nature of the interaction is in general rather limited; only the approximate percentage of the ligand bound is obtained. Other limitations for the use of these two techniques include :

1. binding to the dialysis sac, which may constitute a major obstacle to the use of these techniques.

2. the long time course experiments involving the use of these two methods may cause denaturation of the protein or chemical modification of the drug.

3. disturbances of the equilibrium conditions by factors such as dilution.

4. it is often necessary to correct for, or avoid contribution due to, Donnan effects.

On the other side, fluorimetry and NMR proved to be of great value in protein-binding studies, particularly for investigations on the nature of the binding sites, as already discussed above.

References

1. Martindale The Extra Pharmacopoeia, twenty-ninth edition, p.105, The Pharmaceutical Press, London (1989).

2. Brotzu, G. Ricerche su di un nuovo antibiotico. lavori deU'Instituto D'Igiene di Cagliari, (1948).

3. Abraham, E.P., Loder, P.B.: Cephalosporin C. In E.H.Flynn (Ed.), "Cephalosporins and Penicillins, Chemistry and Biology, Academic Press, New York, pp.1-26 (1972).

4. Abraham, E.P. and Newton, G.G.F., Biochem.J. 79, 377 (1961).

5. Hodgkin, D.C. and Moslen, E.N., Biochem.J. 79, 393 (1961).

6. Morin, R.B., Jackson, B.G., Flynn, E.H., Roeske, R.W., J.Am.Chem.Soc. 84, 3400 (1962).

7. Morin, R.B., Jackson, B.G., Flynn, E.H., Roeske, R.W., Andrews, S.L., J.Am.Chem.Soc. 91, 1396 (1969).

8. Elander, R.P., in "Antibiotics containing the β-lactam structure", A.L.Demain and N.Solomon (Eds.), Part 1, p.101, Springer-Verlag, Berlin (1983).

9. Sigh, P.D., Young, M.G., Johnson, J.H., Cimarusti, C.M. and Sykes. R.B., J.Antibiotics 37, 773 (1984).

10. Chauvette, R.R., Flynn, E.H., Jackson, B.G., Morin, R.B., and associates, J.Am.Chem.Soc. 84, 3401 (1962).

11. Crowfoot, D., Bunn, C.W., Rogers-low, B.W., and Turner-Jones, A., in " The Chemistry of Penicillins", H.T.Clarke, J.R.Johnson, and R.Robinson (Eds.), Princeton University Press, Princeton, N.J., pp.382-414 (1949).

12. Morin, R.B., Jackson, B.G., Mueller, R.A., Lavagnino, E.R., Scanlon, W.B., and Andrews, S.L., J.Am.Chem.Soc. 85, 1896 (1963).

13. Hoover, J.R.E., and Stedman, R.J.A., in " Medicinal Chemistry, 4th ed., Part II ", Burger, A. (Ed.), p.84 (1979).

14. Abraham, E.P., and Newton, G.G.F., Biochem.J. 62, 658 (1956).

15. Abraham, E.P., Newton, G.G.F. and Hale, C.W., Biochem.J. 58, 94 (1954).

16. Jeffery, J.D'A., Abraham, E.P., and Newton, G.G.F., Biochem.J. 75, 216 (1960).

17. Long, A.G., and Turner, A.F., Tetrahedron Letters 7, 421 (1963).

18. Galantay, E., Szabo, A., and Fried, J., Tetrahedron Letters 7, 415 (1963).

19. Galantay, E., Szabo, A., and Fried, J., J.Org.Chem. 29, 3560 (1964).

20. Green, D.M., Long. A.G., May, P.J., and Turner, A.F., J.Chem.Soc. 766 (1964).

21. Meyers, A.I., and Greene, J.M., J.Org.Chem. 31, 556 (1966).

22. Heyme's, R., Amiard, G., and Nomine, G., Compt. Rend. 263, 170 (1966).

23. Woodward, R.B., Heusler, K., Gosteli, J., Naegeli, P., Oppolzer, W., Ramage, R., Ranganathan, S., and Vorbruggen, H., J.Am.Chem.Soc. 88, 852 (1966).

24. Nagarajan, R., Boeck, L.D., Gorman, M., Hamill, R.L., Higgins, C.E., Hoehn, M.M., Starck, W.M. and Whitney, J.G., J.Am.Chem.Soc. 93, 2308 (1971).

25. Stapley, E.O., Antimicrob.Agents and Chemother. 2, 122 (1972).

26. Bergan, T., Drugs 34 (suppl.2), 89 (1987).

27. Flynn,E.H., " Cephalosporins and Penicillins, Chemistry and Biology", p.470, 614, Academic Press, London (1972).

28. Baddiley, J., in "β-Lactam Antibiotics", Salton, M.R.G. and Shockman, G.D. (Eds.), p.13, Academic Press, London (1981).

29. Strominger, J.L. and Tipper, D.J., Am.J.Med. 39, 708 (1965).

30. Lawrence, P.J. and Strominger, J.L., Fed.Proc.Fed.Am.Soc.Exp.Biol. 28, p.473 Abstract 1207 (1969).

31. Spratt, B.G., J. of General Microbiology 129, 1247 (1983).

32. Tipper, D.J., Pharmacol.Therap. 27, 1 (1985).

33. Perlman, D., " Structure-Activity Relationships among the Semisynthetic Antibiotics", p.149, Academic Press, London (1977).

34. Van Heyningen, E., and Ahern, L.K., J.Med.Chem. 11, 933 (1968).

35. Ragnar Norrby, S., Drugs 34 (Suppl.2), 105 (1987).

36. Yamana, T., and Tsuji, A., J.Pharm.Sci. 65, 1563 (1976).

37. Blaha, J.M., Knevel, A.M., Kessler, D.P., Mincy, J.W., and Hem, S.L., J.Pharm.Sci. 65, 1165 (1976).

38. Adriaens, P., Meeschaert, B., Eyssen, H., Roets, E. and Vanderhaeghe, H., J.Chromatogr. 144, 93 (1977).

39. Kessler, D.P., Cushman, M., Ghebre-Sellassie, I., Knevel, A.M., and Hem, S.L., J.Chem.Soc.Perkin Trans.II, 1699 (1983).

40. Das Gupta, V., Can.J.Pharm.Sci. 16, 24 (1981).

41. Sabath, L., Jago, M., and Abraham, E.P., Biochem.J. 96, 739 (1965).

42. Boyd, D.B., in " Chemistry and Biology of β-Lactam Antibiotics, Vol.I", Morin, R.B.,and Gorman, M. (Eds.), 437, Academic Press, New York (1982).

43. Lee, C., Herr, E. and Anderson, R., Clin.Med. 70, 1123 (1963).

44. (a) Hamilton-Miller, J.M., Newton, G.G., Abraham, E.P., Biochem.J. 116, 371 (1970).

(b) Hamilton-Miller, J.M., Richards, E., Abraham, E.P., Biochem.J. 116, 385 (1970).

45. Mozingo, R., and Folkers, K., in "The Chemistry of Penicillin", Clarke, H.T., Johnson, J.R., and Robinson, R. (Eds.),ChapterXVIII, pp.535-656, Princeton Univ. Press, Princeton, N.J. (1949).

46. de Weck, A.L. and Schneider, C.H., J.Antimicrob.Chemother. 6, Suppl.A, 161 (1980).

47. Koneeny, J., Felber, E., and Gruner, J., J.Antibiot. 26, 135 (1973).

48. Schwartz, M.A. and Buckwalter,F.H., J.Pharm.Sci. 51, 1119 (1962).

49. Hou, J.P., and Poole, J.W., J.Pharm.Sci. 60, 503 (1971).

50. Broderson, R., Acta Pharmacol.Toxicol. 3, 345 (1947).

51. Hou, J.P., and Poole, J.W., J.Pharm.Sci. 58, 447 (1969).

52. Schwartz, M.A., J.Pharm.Sci. 58, 643 (1969).

53. Abraham, E.P., in " Topics in Pharmaceutical Sciences ", Perlman D. (Ed.), Vol 1. pp.1-31, Wiley Interscience, New York (1968).

54. Johnson, J.R., Woodward, R.B., and Robinson, R., in " The Chemistry of Penicillin ". Clarke, H.T., Johnson, J.R, and Robinson,R., (Eds.), p.440, Princeton Univ. Press. Princeton, N.J. (1949).

55. Sweet, R.M., and Dahl, L.F., J.Am.Chem.Soc. 92, 5489 (1970).

56. Hermann, R.B., J.Antibiot. 26, 223 (1973).

57. Boyd, D.B., Hermann, R.B., Presti, D.E., and Marsh, M.M., J.Med.Chem. 18, 408 (1975).

58. Pollock, M.R., in "Antimicrobial Agents and Chemotherapy ", Sylvester J.C. (Ed.). pp.292-301, Williams & Wilkins, Baltimore, Maryland (1965).

59. Faraci, W.S., and Pratt, R.F., J.Am.Chem.Soc. 106, 1489 (1984).

60. Page, M.I., Proctor, P., J.Am.Chem.Soc. 106, 3820 (1984).

61. Clarke, H.T., Johnson, J.R., and Robinson, R. (Eds.), " The Chemistry of Penicillin ". Princeton Univ. Press, Princeton, N.J., (1949).

62. Degelaen, J.P., Loukas, S.L., Feeney, J., Roberts, G.C., and Burgen, A.S.. J.Chem.Soc.Perkin Trans.II, 86 (1979).

63. Rattie, E.S., Zimmerman, J.J., and Ravin, L.J., J.Pharm.Sci. 68, 1369 (1979).

64. Tsuji, A., Yamana, T., Nakashima, E., Deguchi, Y., Nishide, K., Shimizu, T.. Horiuchi, S. and Ishikawa, K., J.Pharm.Sci. 70, 1120 (1981).

65. Indelicato, J.M., Norvilas, T.T., and Wheeler, W.J., J.Chem.Soc.Chem.Comm., 1162 (1972).

66. Indelicato, J.M., Norvilas, T.T., Pfeiffer, R.R., Wheeler, W.J., and Wilham, W.L., J.Med.Chem. 17, 523 (1974).

67. Dinner, A., J.Med.Chem. 20, 963 (1977).

68. Coene, B., Schanck, A., Dereppe, J.M., and Meerssche, M.V., J.Med.Chem. 27, 694 (1984).

69. Hall, D.A., J.Pharm.Sci. 62, 981 (1973).

70. Namiki, Y., Tanabe, T., Toshimitsu, K., Tanabe, J., Okimura, Y., Koda, S. and Morimoto, Y., J.Pharm.Sci. 76, 208 (1987).

71. Mangia, A., Silingardi, S., Bortesi, F., Grisanti, G. and Di Bitetto, M., J.Pharm.Sci. 68, 652 (1979).

72. Indelicato, J.M., Engel, G.L., and Occolowitz, J.L., J.Pharm.Sci. 74, 1162 (1985).

73. Bundgaard, H., Arch.Pharm.Chemi.Sci.Ed. 4, 25 (1975).

74. Cohen, A.I., Funke, P.T., and Puar, M.S., J.Pharm.Sci. 62, 1559 (1973).

75. Fabre, H., Hussam-Eddine, N., and Berge, G., J.Pharm.Sci. 73, 611 (1984).

76. Fabre, H., and Blanchin, M.D., Analyst Vol.III, 1281 (1986).

77. Indelicato, J.M., Wilham, W.L., and Cerimele, B.J., J.Pharm.Sci. 65, 1175 (1978).

78. Fabre, H., and Hussam-Eddine, N., J.Pharm.Pharmacol. 34, 425 (1982).

79. Bornstein, M., Klink, P.R., Farrell, B.T., Winely, C.L., and Boylan, J.C., Am.J.Hosp.Pharm. 37, 98 (1980).

80. Hashimoto, N., Tasaki, T., and Tanaka, H., J.Pharm.Sci. 73, 369 (1984).

81. Hashimoto, N., and Tanaka, H., J.Pharm.Sci. 74, 68 (1985).

82. Hashimoto, N., and Ichihashi, T., Pharm.Res. 5, 266 (1988).

83. Branch, S.K., Casy, A.F., and Ominde, M.A., J.Pharm.Biomed.Anal. 5. 73 (1987).

84. Branch, S.K., Casy, A.F., Lipczynski, A. and Ominde, M.A., Magn.Res.Chem. 24, 465 (1986).

85. Lipczynski, A.M., Ph.D. Thesis : The degradation of benzylpenicillin in aqueous solution, University of Bath (1988).

86. Neidleman, S.L., Pan, S.C., Last, J.A., and Dolfini, J.E., J.Med.Chem. 13, 386 (1970).

87. Mobashery, S., and Johnston, M., J.Org.Chem. 51, 4723 (1986).

88. Kukolja, S.,Pfeil, J.L., Draheim, S.C., and Ott, J.L., J.Med.Chem. 1903 (1985).

89. Lammert, S.R., Ellis, A.I., Chauvette, R.R., and Kukolja, S., J.Org.Chem. 43, 1243 (1978).

90. Demarco, P.V., and Nagarajan, R., in E.H.Flynn (Ed.) : " Cephalosporins and Penicillins, Chemistry and Biology ", Chapter 8, p.330, Academic Press, New York (1972).

91. Roets, E.E., Hoogermartens, J.H., and Vanderhaeghe, H.J., J.Assoc.Off.Anal.Chem. 64, 166 (1981).

92. Green, G.F.H., Page, J.E., Staniforth, S.E., Chem.Commun., 597 (1966).

93. Green, G.F.H., Page, J.E., and Staniforth, S.E., J.Chem.Soc., 1595 (1965).

94. Schanck, A., Coene, B., Van Meerssche, M., and Dereppe, J.-M., Org.Magn.Res. 12, 337 (1979).

95. Benn, R., and Gunther, H., Angew.Chem.Inst.Ed.Engl. 22, 350 (1983).

96. Vold, R.L., Waugh, J.S., Klein, M.P., and Phelps, D.E., J.Chem.Phys. 48, 3831 (1968).

97. McMillan, I., and Stoodly, R.J., Tetrahedron Lett. 11, 1205 (1966).

98. Barrow, K.D., and Spotswood, T.M., Tetrahedron Lett., 3325 (1965).

99. Abraham, R.J., and Loftus, P., "^{1}H and ^{13}C NMR Spectroscopy", p.46, Heyden, London (1981).

100. Williams, D.H., and Fleming," Spectroscopic methods in Organic Chemistry ", pp.108, 141, and 144, McGraw-Hill, London (1980).

101. Tori, K., Nishikawa, J., and Takeuche, Y., Tetrahedron Lett. 22, 2793 (1981).

102. Paschal, J.W., and Dorman, D.E., J.Org.Chem. 43, 2013 (1978).

103. Dereppe, J.M., Schanck, A., Coene, B., Moreau, C., and Van Meerssche, M., Org.Magn.Reson. 11, 638 (1978).

104. Hansen, P.E., Prog.Nucl.Magn.Reson.Spectrosc., edited by J.W.Emsley, J.Feeney, and L.H. Sutcliffe, Pergamon Press, Oxford, 14, 175 (1981).

105. Neuss, N., Nash, C.H., Lemke, P.A., and Grutzner, J.B., J.Am.Chem.Soc. 93, 2337 (1971).

106. Neuss, N., Proc.Roy.Soc.Ser.(London) B179, 335 (1971).

107. Wehrli, F.W., and Wirthlin, T., Interpretation of 13C NMR spectra, p.134, Heyden, London (1976).

108. Wehrli, F.W., Topics in 13C NMR Spectroscopy, Vol.2, p.343 (1976), and references therein.

109. Terui, Y., Tori, K., Maeda, S., Sawa, Y.K., Tetrahedron Lett., 2853 (1975).

110. Mondelli, R., and Ventura, P., J.Chem.Soc.Perkin Trans II. No.9-15, 1749 (1977).

111. Stothers, J.B., Carbon-13 NMR Spectroscopy, p.148, Academic Press, New York (1972).

112. (a) Cooper, P.D., Bacteriol.Rev. 20, 28 (1956);

(b) Collins, J.F., and Richmond, M.H., Nature 195, 142 (1962);

(c) Cocker, J.D., Eardley, S., Gregory, G.I., Hall, M.E., and Long, A.G., J.Chem.Soc. C, 1142 (1966); and

(d) Morin, R.B., Advan.Drug Res. 4, 48 (1967).

113. Sternhell, S., Quart.Rev.Chem.Soc. 23, 236 (1969).

114. Benedict,R.G., Schmidt,W.H., Coghill,R.D., and Oleson,A.P., J.Bact. 49, 85 (1945).

115. Rapson,H.C., and Bird,A.E., J.Pharm.Pharmacol.Suppl. 15. 222T (1963).

116. Peck,R.L., and Folkers,K., in reference 61, Chapter VII, pp.144-206.

117. Woodward,R.B., Neuberger,A., and Trenner,N.R., in reference 61, Chapter XIV, pp.415-436.

118. Dennen,D.W., and Davis,W.W., " Antimicrobial Agents and Chemotherapy ", American Society for Microbiology, Ann Arbor, Mich., p.531 (1961).

119. Yamana,T., Tsuji,A., Kiya,E. and Miyamoto,E., J.Pharm.Sci. 66, 861 (1977).

120. Proctor,P., Gensmantel, N.P. and Page,M.I., J.Chem.Soc.Perkin Trans. II, 1185 (1982).

121. Ghebre-sellassie,I., Hem,S.L. and Knevel,A.M., J.Pharm.Sci. 73, 125 (1984).

122. Ressler, C., Neag, P.M., and Mendelson, L.M., J.Pharm.Sci. 74, 448 (1985).

123. (a) DeWeck, A.L., and Bhem, G., Internat.Arch.Allergy Appl.Immunol. 27, 221 (1965).
(b) Schneider, C.H., and DeWeck, A.L., Nature 208, 57 (1965).

124. (a) Morris, J.M., and Page, M.I., J.Chem.Soc.Perkin Trans. 2, 21 (1980).
(b) Morris, J.M., and Page, M.I., J.Chem.Soc.Perkin Trans. 2, 220 (1981).

125. Jones, I.F., Page, J.E., and Rhodes, C.T., J.Pharm.Pharmacol. 20, 45 (1968).

126. Wright, W.E., Frogge, J.A., Antimicrob.Ag.Chemother. 17, 99 (1980).

127. Kukolja, S., J.Med.Chem. 11, 1067 (1968).

128. Berge, S.M, Henderson. N.L., Frank, M.J., J.Pharm.Sci. 72, 59 (1983).

129. Gould, E.S., " Mechanism and Structure in Organic Chemistry ", Holt, Rinehart, and Winston, New York, pp.314-322 (1959).

130. Cocker, J.D., Cowley, B.R., Cox, J.S., Eardley, S., Gregory, G.I., Lazenby, J.K., Long, A.G., Sly, J.C. and Somerfield, G.A., J.Chem.Soc., 1959 (1967).

131. Taylor, A.B., J.Chem.Soc., 7020 (1965).

132. Hatfield, L.D., Fisher, J.W., Dunigan, J.M., Burchfield, R.W., Greene, J.M., Webber, J.A., Vasileff, R.J. and Kinnick, M.D., Philos.Trans.Roy.Soc.Lond.B : Biol.Sci. 289, 173 (1980).

133. Newton, G.G., Abraham, E.P., and Kuwabara, S., Antimicrob.Ag.Chemother. 1967, 449 (1968).

134. Ross, G.W., and O'Callaghan, C.H., in reference 149, pp.69-85 (1975).

135. O'Callaghan, C.H., Morris, A., Kirby, S.M., and Shingler, A.H., Antimicrob.Ag.Chemother. 1, 283 (1972).

136. Eggers, S.H., Kane, V.V., and Lowe, G., J.Chem.Soc., 1262 (1965).

137. Gorman, M., Ryan, C.W., in reference 27, pp.544-569.

138. Sassiver, M.L., Lewis, A., in reference 33. pp.87-160.

139. Boyd, D.B., Herron, D.K., Lunn, W.H., Spitzer, W.A., J.Am.Chem.Soc. 102, 1812 (1980).

140. O'Callghan, C.H., Kirby, S.M., Morris, A., Waller, R.E., and Duncombe, R.E., J.Bacteriol. 110, 988 (1972).

141. Waller, R.E., Analyst 98, 535 (1973).

142. Bundgaard, H., Arch.Pharm.Chemi., Sci.Ed. 3, 94 (1975).

143. Boyd, D.B., Lunn, W.H., J.Med.Chem. 22, 778 (1979).

144. Grabowski, E.J., Douglas, A.W., and Smith, G.B., J.Am.Chem.Soc. 107, 267 (1985).

145. Yamana, T., Tsuji, A., Kanayama, K., and Nakano, O., J.Antibiot. 27, 1000 (1974).

146. Dinner, A., Indelicato, J.M., Peters, L.R., and Wilham, W.L., J.Med.Chem. 20, 961 (1977).

147. Richmond, M.H., and Sykes, R.B., Advan.Microb.Physiol. 9, 31 (1973).

148. Datta, N., and Richmond, M.H., Biochem.J. 98, 204 (1966).

149. Ross, G.W. and o'Callaghan, C.H., in " Methods in Enzymology, Vol XLIII : Antibiotics ", Hash, J.H. (Ed.), p.69, Academic Press, New York (1975).

150. Livermore,A.H., Carpenter,F.H., Holley,R.W., and du Vingeaud,V., J.Biol.Chem. 175, 721 (1948).

151. Levine,B.B., Arch.Biochem.Biophys. 93, 50 (1961).

152. deKoning, J.J., Kooreman, H.J., Tan, H. and Verweij, J., J.Org.Chem. 40, 1346 (1975).

153. Pratt, R.F., and Faraci, W.S., J.Am.Chem.Soc. 108, 5328 (1986).

154. Boyd, D.B., J.Org.Chem. 50, 886 (1985).

155. Page 117 in reference 13

156. British Pharmacopoeia, Vol.II, A53 (1980).

157. Goldstein, A., Aronow, L., and Kalman, S.M., " Principles of Drug Action : The Basis of Pharmacology ", pp.1, 14, 18, 158, John Wiley and sons, London (1974).

158. Bridges, J.W. and Wilson, A.G.E., in " Progress in Drug Metabolism, Vol 1 ", Bridges, J.W. and Chasseaud, L. (Eds.), pp.193-247 (1976).

159. Brodei, B.B., and Hogben, C.A.M., J.Pharm.Pharmacol. 9, 345 (1957).

160. Bowman, W.C., Rand, M.J., and West G.B., " Textbook of Pharmacology ", 6^{th} ed., Chapters 39 and 40 (1974).

161. Goldstein, A., Pharmacol.Rev. 1, 102 (1949).

162. Craig, W.A. and Welling, P.G., Clin.Pharmacokin. 2, 252 (1977).

163. Gourley, D.R., Modern Trends in Pharmacol. and Ther. 1, 1 (1967).

164. Kunin, C.M., Clin.Pharmac.Ther. 7, 180 (1966).

165. Brodie, B.B., Proc.R.Soc.Med. 58, 946 (1965).

166. White, A., Handler, P. and Smith, E.G., " Principles of Biochemistry ", McGraw-Hill, New York (1968).

167. Jonas, A. and Weber, G., Biochemistry 10, 1335 (1971).

168. Thorp, J.M., in Binns, T.B. (Ed.), " Absorption and distribution of drugs ", pp.64-76, E.S.Livingstone, London (1964).

169. Sudlow, G., Birkett, D.J. and Wade, D.N., Molec.Pharmacol. 11, 824 (1975).

170. Kiem, E., Fehske, K.J., and Muller, W.E., Wld.Rev.Nutr.Diet. 43, 179 (1984).

171. Klotz, I.M., Ann.N.Y.Acad.Sci. 226, 18 (1973).

172. Nagwekar, J.B. and Kostenbauder, H.B., J.Pharm.Sci. 59, 751 (1970).

173. Wise, R. and Gillet, A.P., J.Infect.Dis. 142, 77 (1980).

174. Selwyn, S., The Lancet 1, 1104 (1978).

175. Briand, C., Sarrazin, M. and Peyrot, V., Molec.Pharmacol. 21, 92 (1982).

176. Barza, M., Weinstein, L., Ann.N.Y.Acad.Sci. 235, 613 (1974).

177. Nightingale, D., Greene, D.S. and Quintilliani, R., J.Pharmacol.Sci. 64, 1899 (1975).

178. Singhvi, S.M., Reald, A.F. and Leitz, M., J.Lab.Clin.Med. 89, 414 (1977).

179. Csiba, A., Ludwig, E. and Magyar, T., Gyogyszereszet 29, 251 (1985).

180. Veronese, F.M., Bevilacqua, R., Boccu, E. and Benassi, C.A., Farmaco-Ed.Sci. 32, 303 (1977).

181. Jun, H.W., Mayer, R.T., Himel, C.M. and Luzzi, L.A., J.Pharm.Sci. 60, 182 (1971).

182. Chignell, C.F., Molec.Pharmacol. 5, 244 (1969).

183. Sudlow, G., Birkett, D.J. and Wade, D.N., Molec.Pharmacol. 9, 649 (1973).

184. Muller, W.E. and Wolbert, U., Biochem.Pharmacol. 25, 141 (1976).

185. Kim. C.K., Yang, J.S. and Lim, Y.S., Arch.Pharm.Res. 6, 55 (1983).

186. Kim. C.K., Yang, J.S. and Lim, Y.S., Arch.Pharm.Res. 7, 11 (1984).

187. Jardetzky, O., Adv.Chem.Phys. 7, 499 (1964).

188. Jardetzky, O. and Wade-Jardetzky, N.G., Molec.Pharmac. 1, 214 (1965).

189. Fischer, J.J. and Jardetzky, O., J.Amer.Chem.Soc. 87, 3237 (1965).

190. Meyer, M.C. and Guttman, D.E., J.Pharm.Sci. 57, 895 (1968).

191. Chignell, C.F., "Methods in Pharmacology, Physical Methods", Vol.2, Appleton-Century-Croft, New York, pp.1-61 (1972).

192. Goldstein, A., Pharmacol.Rev. 1, 102 (1949).

193. Kurz, H., Trunk, H. and Weitz, B., Arzneimitt.-Forsch. 27, 1373 (1977).

194. Giacomini, K.M., Abang, A. and Blaschke, T.F., Bri.J.Clin.Pharmacol. 14, 752 (1982).

195. Abraham, R.J. and Loftus, P., "Proton and Carbon-13 NMR Spectroscopy, An integrated approach, Heyden and Son, pp.84-86, pp.121-125 and 139 (1980).

196. Karush, F., J.Amer.Chem.Soc. 72, 2705 (1950).

197. Scatchard, G., Ann.N.Y.Acad.Sci. 51, 660 (1949).

198. Klotz, I.M., Walker, F.M. and Pivan, R.B., J.Amer.Chem.Soc. 68, 1486 (1946).

199. Keen, P., in " Handbook of Experimental Pharmacology. Concepts in Biochemical Pharmacology 28 (Part I) ", Brodie, B.B. and Gillette, J.R.(Eds.), pp.213-233, Springer-Verlag, New York, N.Y. (1971).

200. Willard, H.H., Merritt, L.L. and Dean, J.A., " Instrumental Methods of Analysis ", 5^{th} ed., D.Van nostrad company, New York, 133 (1974).

201. Chignell, C.F., Molec.Pharmacol. 6, 1 (1970).

202. Steiner, R.F., J.Biol.Chem. 241, 560 (1966).

203. Brand, L., Gohlke, J.R. and Rao, S., J.Biochem. 6, 3510 (1967).

204. Mclure, W.O. and Edelman, G.M., Biochemistry 5, 1908 (1966).

205. Stryer, L., J.Mol.Biol. 13, 482 (1965).

206. Geisow, M.J. and Beaven, G.H., Biochem.J. 163, 477 (1977).

207. Freeman, R., Hill, H.D.W., J.Chem.Phys. 51, 3140 (1969).

208. Hsu, P.L., Ma, J.K. and Jun, H.W., J.Pharmacol.Sci. 63, 27 (1974).

209. Perrin, D.D. and Dempsey, B., "Buffers for pH and Metal Ion Control", p.77, Chapman and Hall, London (1974).

210. Rosenburg, R.M. and Klotz, I.M., in Alexander, P. and Block, R.J. (Eds.), " A laboratory manual of analytical methods of protein chemistry, Vol.2, pp.131-168 (1960).

211. Bennett, J.V. and Kirby, W.M.M., J.Lab.Clin.Med. 66, 721 (1965).

212. Steinberg, I.Z. and Schachman, H.K., Biochemistry 5, 3728 (1966).

213. Wood, G.C. and Cooper, P.F., Chromatog.Rev. 12, 88 (1970).

214. O'Reilly, R.A. and Kowitz, P.E., J.Clin.Invest. 46, 829 (1967).

215. Chignell, C.F., in reference 191, pp.33-61.

216. Edelman, G.M. and McClure, W.O., Acc.Chem.Res. 1, 65 (1968).

217. Chen, R.F., J.Biol.Chem. 242, 5813 (1967).

218. Klotz, I.M., Triwush, H. and Walker, F.M., J.Am.Chem.Soc. 70, 2935 (1948).

219. Brand, l. and Gohlke, J.R., Ann.Rev.Biochem. 41, 843 (1972).

220. Javidon, S. and Mrtek, R.G., J.Pharm.Sci. 62, 420 (1973).

221. Sharples, D., J.Pharm.Pharmac. 28, 100 (1976).

222. Edsall, J.T. and Wyman, " Biophysical Chemistry ", Academic Press Inc., New York, N.Y., p.534 (1958).

223. Jardetzky, O. and Jardetzky, C.D., in " Methods of Biochemical Analysis ", Glick, D. (Ed.), IX, p.235, Interscience Publ., New York (1962).

224. Cohen, M. and Hughes, T.R., Jr., J.Biol.Chem. 237, 176 (1962).